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(54) Title: THE USE OF PROCYANIDINS IN THE MODULATION OF CYTOKINE GENE EXPRESSION AND PROTEIN SECRETION

(57) Abstract: Cocoa procyanidins and cocoa extracts which include procyanidin monomers and their oligomers are useful in the modulation of an immune response in a mammal and the modulation of an inflammatory response in a mammal. The cocoa procyanidins and liquid or dry cocoa extracts can be included in foods, food supplements and pharmaceuticals for the modulation of cytokine gene product and protein levels and for providing beneficial effects to subjects suffering from asthma or viral infections or at risk of viral infections.

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**The Use Of Procyanidins In The Modulation of Cytokine Gene Expression  
and Protein Secretion**

5

**FIELD OF THE INVENTION**

This invention relates to nutritional and medical uses of cocoa polyphenols and extracts containing cocoa polyphenols including cocoa procyanidins.

**BACKGROUND OF THE INVENTION**

10 Polyphenols are an incredibly diverse group of compounds (Ferreira *et al.*, 'Diversity of Structure and Function in Oligomeric Flavanoids, Tetrahedron , 48:10, 1743-1803, 1992). They widely occur in a variety of plants, some of which enter into the food chain. In some cases they represent an important class of compounds for the human diet. Although some of the polyphenols are considered to be non-nutritive,  
15 interest in these compounds has arisen because of their possible beneficial effects on health.

For instance, quercetin (a flavonoid) has been shown to possess anticarcinogenic activity in experimental animal studies (Deshner *et al.*, 'Quercetin and Rutin as Inhibitors of Azoxymethanol-induced Colonic Neoplasia',  
20 Carcinogenesis, 7:1193-1196, 1991; and Kato *et al.*, 'Inhibition of 12-O-tetradecanoylphorbol-13-acetate Induced Tumor Promotion and Ornithine Decarboxylase Activity by Quercetin: Possible Involvement of lipoxygenase Inhibition' , Carcinogenesis, 4, 1301-1305 1983). (+)-catechin and (-)-epicatechin (flavan-3-ols) have been shown to inhibit Leukemia virus reverse transcriptase  
25 activity (Chu *et al.*, 'Inhibitory Effects of Flavonoids on Maloney Murine Leukemia Virus Reverse Transcriptase Activity, J. of Natural Products, 55:2, 179-183, 1992). Nobotanin (an oligomeric hydrolyzable tannin) has also been shown to possess anti-tumor activity (Okuda *et al.*, 'Molecular Structures and Pharmacological Activities of Polyphenols - Oligomeric Hydrolyzable Tannins and Others' - Presented at the XVI<sup>th</sup>  
30 International Conference of the Groupe Polyphenols, Lisbon, Portugal, July 13-16, 1992). Statistical reports have also shown that stomach cancer mortality is significantly lower in the tea producing districts of Japan. Epigallocatechin gallate

has been reported to be the pharmacologically active material in green tea that inhibits mouse skin tumors (Okuda *et al.*, 'Molecular Structures and Pharmacological Activities of Polyphenols – Oligomeric Hydrolyzable Tannins and Others. Presented at the XVIth International Conference of the Groupe polyphenols, Lisbon, Portugal, 1992). The antioxidant properties of cocoa bean extract, thought to contain epicatechin and analogous compounds, has been reported to be responsible for inhibiting formation of gastric ulcers in rats by Osakabe *et al.* (JP 7274894 "Food and Drink For Preventing Gastric Ulcers – Contains Antioxidation Substance Extracted from Cacao Beans using Hot Water or Ethanol" Oct. 1995; JP 7213251 "Method of Manufacturing an Antioxidation Substance and a Health Food or Drink Item Containing an Antioxidation Substance" Aug. 1995). Ellagic acid has also been shown to possess anticarcinogen activity in various animal tumor models (Boukharta *et al.*, Efficacy of Ellagitannins and Ellagic Acid as Cancer Chemopreventive Agents - Presented at the XVI<sup>th</sup> International Conference of the Groupe Polyphenols, Lisbon, Portugal, July 13-16, 1992). Lastly, proanthocyanidin oligomers have been reported by the Kikkoman Corporation for use as antimutagens ('Antimutagenic Agent Containing Proanthocyanidin Oligomer Preferably Having Flavan-3-ol-diol Structure' JP 04190774A, July 7, 1992). Indeed, the area of phenolic compounds in foods and their modulation of tumor development in experimental animal models has been recently presented to the 202<sup>nd</sup> National Meeting of The American Chemical Society (Phenolic Compounds in Foods and Their Effects on Health II. Antioxidants & Cancer Prevention, Huang, M.-T., Ho, C.-T., and Lee, C.Y. editors, ACS Symposium Series 507, American Chemical Society, Washington, D.C. 1992).

Recent findings, discussed below, suggesting that cocoa polyphenols may have an immunomodulatory effect on human peripheral blood mononuclear cells (PBMCs), prompted a series of experiments on the effects of the purified procyanidin oligomers on cytokine gene expression and protein secretion from unstimulated and stimulated PBMCs. The results were unexpected. Because it has been reported that IL-1, a macrophage derived factor, is needed in the stimulation of IL-2 production from T-cells (Farrar *et al.*, "Macrophage-independent activation of helper T-cells. Production of Interleukin-2" J. Immunol. 125:793, 1980), the discovery, reported

herein, that IL-1 $\beta$  protein production and secretion by PBMCs is up-regulated by *in vitro* treatment with cocoa polyphenols, was not anticipated.

Sato *et al.* have shown that polyphenols from other plant sources can inhibit the cellular expression of interleukin-8 and monocyte chemoattractant-1 when induced by the pro-inflammatory cytokine tumor necrosis factor- $\alpha$  (J. Rheumatol. 24 1680-84, 1997). Two studies have investigated the effects of cocoa polyphenols on the transcription of IL-2 in PBMCs *in vitro* (Mao *et al.*, "The Influence of Cocoa Procyanidins on the Transcription of Interleukin-2 in Peripheral Blood Mononuclear Cells", Int. J. Immunotherapy, xv(1) 23-29, 1999; and Sanbongi *et al.*, "Polyphenols 10 in Chocolate, Which Have Antioxidant Activity, Modulate Immune Functions in Humans *in vitro*", Cell. Immunol. 177, 129-136, 1997). In both cases the results showed that treating resting PBMCs with cocoa polyphenols inhibited IL-2 mRNA transcription. The Sanbongi study also showed that IL-2 secretion from T-cells was also inhibited by *in vitro* treatment with cocoa polyphenols.

#### 15 SUMMARY OF THE INVENTION

A composition which comprises monomers and/or oligomers of cocoa procyanidins is useful for modulating cytokine production levels in human peripheral blood monocytes and inflammatory pathways in a mammal. Monomers and/or oligomers of cocoa procyanidins may be in a form of a cocoa extract, fractions 20 thereof containing at least one procyanidin monomer or oligomer, or synthetically prepared counterparts thereof, in liquid or dry form. They also may be in a form of a cocoa product processed in a way to preserve the levels of cocoa procyanidins, for example, cocoa solids and chocolate liquor having conserved levels of cocoa procyanidins. Finally, derivatives including methylated metabolites of cocoa 25 procyanidins are also useful. For brevity and ease of review of the disclosure herein, any reference to cocoa procyanidins encompasses monomers and/or oligomers. A person of skill in the art will understand that the compounds described herein are also referred to in the art as flavanols and the term cocoa procyanidin is also used to refer to oligomers only.

30 In one aspect, the invention relates to the use of the compounds described herein for the manufacture of a medicament, food or dietary supplement for treating

asthma or for improving the respiratory tract pathology associated with asthma. Thus, a method for treating asthma, or for improving the respiratory tract pathology associated with asthma, by administering an effective amount of the compound(s) of the invention to a mammal, such as a human or a veterinary animal, is provided.

5 In another aspect, the invention relates to the use of the compound(s) described herein for the manufacture of a medicament, food or dietary supplement for preventing a viral infection, or for reducing the risk of a viral infection in a subject exposed to a virus. The subject is a mammal such as a human or a veterinary animal. Thus, a method for preventing a viral infection, or for reducing the risk of a viral  
10 infection, in a mammal by administering to the mammal an effective amount of the compound(s) of the invention is provided.

In yet another aspect, the invention relates to the use of the compound(s) described herein for the manufacture of a medicament, food or dietary supplement for treating a viral infection, for enhancing an immune response to a viral infection or for  
15 reducing the symptoms of a viral infection, in a subject infected with a virus. The subject is a mammal such as a human or a veterinary animal. Thus, a method for treating a viral infection, for enhancing an immune response to a viral infection or for reducing symptoms of a viral infection, in a mammal by administering to the mammal the compound(s) of the invention is provided.

20 The cocoa extract may be obtained from cocoa beans, cocoa nibs, cocoa nib fractions, chocolate liquor, and/or partially defatted or fully defatted cocoa solids by extracting with a solvent which dissolves the cocoa polyphenol such as water, selected organic solvents, or mixtures thereof. Organic solvents include ketones such as acetone or methyl ethyl ketone or alcohols such as ethanol or methanol.

25 The crude cocoa extract may be purified by gel permeation chromatography to remove the xanthine alkaloids caffeine and theobromine. Alternatively, the caffeine can be extracted with chlorine if an aqueous solvent mixture is used, the theobromine will precipitate out after cooling, and can be filtered off. The purified extract may be further purified and fractionated by preparative high performance liquid  
30 chromatography (HPLC). Preferably, the extract is purified and fractionated by gel permeation chromatography followed by preparative high performance liquid chromatography (HPLC). The purified liquid extract may be fractionated into sub-

fractions containing substantial amounts of specific procyanidin monomers and/or oligomers, and the sub-fractions may be further purified using the procedures discussed above. The substantially pure extract can be enriched by adding the oligomeric sub-fractions discussed above. The procyanidin monomers are mainly  
5 epicatechin, but some catechin is present.

The compositions comprising high cocoa polyphenol solids and/or the cocoa extract, sub-fractions thereof, synthetically prepared compounds, derivatives including methylated metabolites thereof or mixtures thereof may further comprise a liquid or a solid carrier suitable for use in foods, food supplements or  
10 pharmaceuticals. Such products include food and beverage products, in addition to capsule, tablet and pressed powder compositions. The compound(s) of the invention may also be adapted for nasal administration, *i.e.*, they may be contained in a dosage form for nasal administration such as an inhaler, nebulizer, nasal drops or nasal spray.

For the purposes of this application, the following definitions will enable a  
15 clearer understanding of what is disclosed and claimed:

As used herein a "food" is a material consisting essentially of protein, carbohydrate and/or fat, which is used in the body of an organism to sustain growth, repair and vital processes and to furnish energy. Foods may also contain supplementary substances such as minerals, vitamins and condiments. See Merriam-  
20 Websters Collegiate Dictionary, 10<sup>th</sup> Edition, 1993.

As used herein, a "pharmaceutical" is a medicinal drug. See Merriam-Websters Collegiate Dictionary, 10<sup>th</sup> Edition, 1993.

As used herein, a "Food Supplement" is a product (other than tobacco) that is intended to supplement the diet that bears or contains one or more of the following  
25 dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract or combination of these ingredients. The food supplement is also referred to herein as a dietary supplement.

The compositions comprising the cocoa extracts, sub-fractions thereof,  
30 synthetically prepared compounds thereof, cocoa procyanidin derivative and high cocoa polyphenol solids or mixtures thereof, are useful for modulating cytokine production levels in human peripheral blood monocytes and inflammatory pathways

in a mammal. In addition to asthma and anti-viral applications, diseases, such as inflammatory bowel disease, arthritis, edema, gingivitis and periodontitis, which are caused by chronic inflammation, are also prevented.

#### BRIEF DESCRIPTION OF THE DRAWINGS

5       The following Detailed Description will be better understood by reference to the accompanying drawings wherein:

Fig. 1 shows a purification scheme for the isolation of procyanidins from cocoa.

10       Fig. 2 is a schematic diagram the Experimental Design of the cytokine modulation studies.

Fig. 3 shows a representative gel permeation chromatogram from the fractionation of crude cocoa procyanidins.

Fig. 4 shows a representative reverse-phase HPLC chromatogram showing the separation (elution profile) of cocoa procyanidins extracted from unfermented cocoa.

15       Fig. 5 shows a representative normal phase HPLC separation of cocoa procyanidins extracted from unfermented cocoa.

Fig. 6 shows a MALDI-TOF mass spectrum of cocoa procyanidin oligomers (tetramers to octadecamers).

20       Fig. 7 shows the effects of cocoa procyanidin fractions on the transcription of IL-1 $\beta$  from human PBMCs.

Fig. 8 shows the effects of cocoa procyanidin fractions on the transcription of IL-1 $\beta$  from PHA-stimulated human PBMCs.

Fig. 9 shows the effects of cocoa procyanidin fractions on the protein secretion of IL-1 $\beta$  from human PBMCs.

25       Fig. 10 shows the effects of cocoa procyanidin fractions on the protein secretion of IL-1 $\beta$  from PHA-stimulated human PBMCs.

Fig. 11 shows the effects of cocoa procyanidin fractions on the expression of IL-2 in PHA-stimulated PBMCs from 4 subjects (c-f).

30       Fig. 12 shows the effects of cocoa procyanidin fractions on the expression of IL-4 in resting human PBMCs (12a) and in PHA-stimulated PBMCs (12b).

Fig. 13 shows the effects of cocoa procyanidin fractions on IL-4 protein secretion from resting PBMCs.

Fig. 14 shows the effects of cocoa procyanidin fractions on IL-4 protein secretion from PHA-stimulated PBMCs.

5 Fig. 15 shows the effects of cocoa procyanidin fractions on the expression of IL-6 in resting human PBMCs (15a) and in PHA-stimulated PBMCs (15b).

Fig. 16 shows the effects of cocoa procyanidin fractions on IL-6 protein secretion from resting PBMCs (16a) and from PHA-stimulated PBMCs (16b).

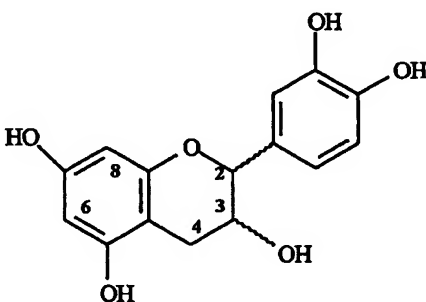
Fig. 17 shows the effects of cocoa extract on TNF- $\alpha$  production in PBMC's.

10

### DETAILED DESCRIPTION

Cocoa procyanidins (monomers and/or oligomers) are useful for modulating cytokine production levels in a mammal and are therefore useful for preventing, treating and/or alleviating the pathology associated with inflammatory pathways. In certain embodiments of the invention, these compounds are administered to subjects,  
15 such as humans or veterinary animals, that suffer from asthma and/or viral infections or that are exposed to viral particles, *i.e.*, are at risk of a viral infection.

Monomers comprising procyanidins have the structure:



20 Procyanidins include those found in cocoa beans obtained from *Theobroma cacao* and various related cocoa species, as well as the genus *Herrania* and their inter- and intra-genetic crosses.

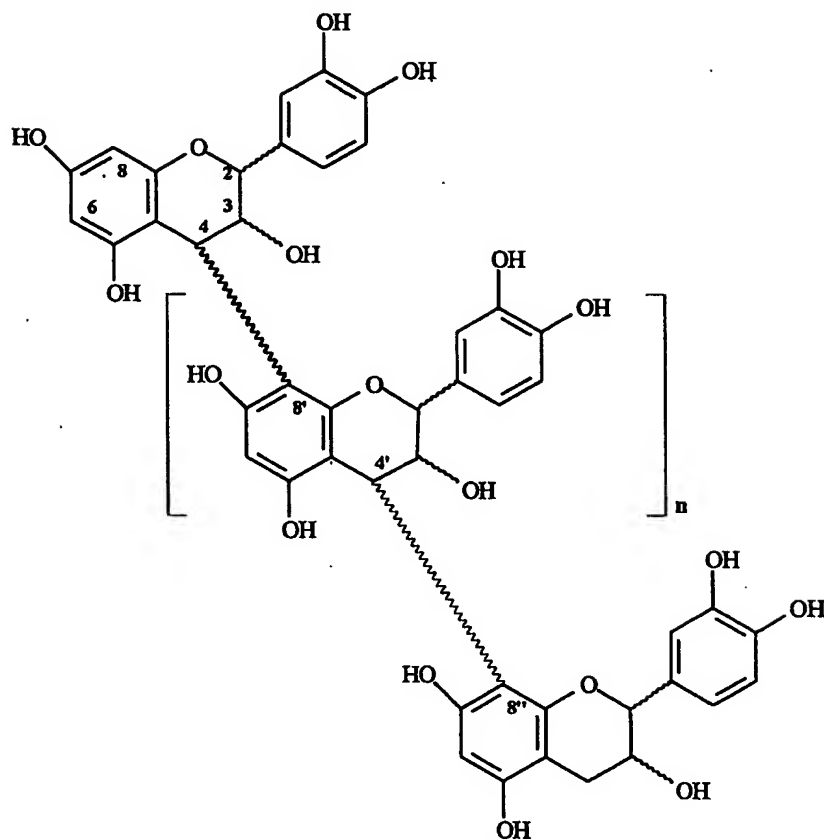
Monomers comprising procyanidins include (+)-catechin, (-)-epicatechin and their respective epimers (e.g. (-)-catechin and (+)-epicatechin).

25 Synthetic linear and/or branched oligomers having the following structures are illustrative of the cocoa procyanidins.

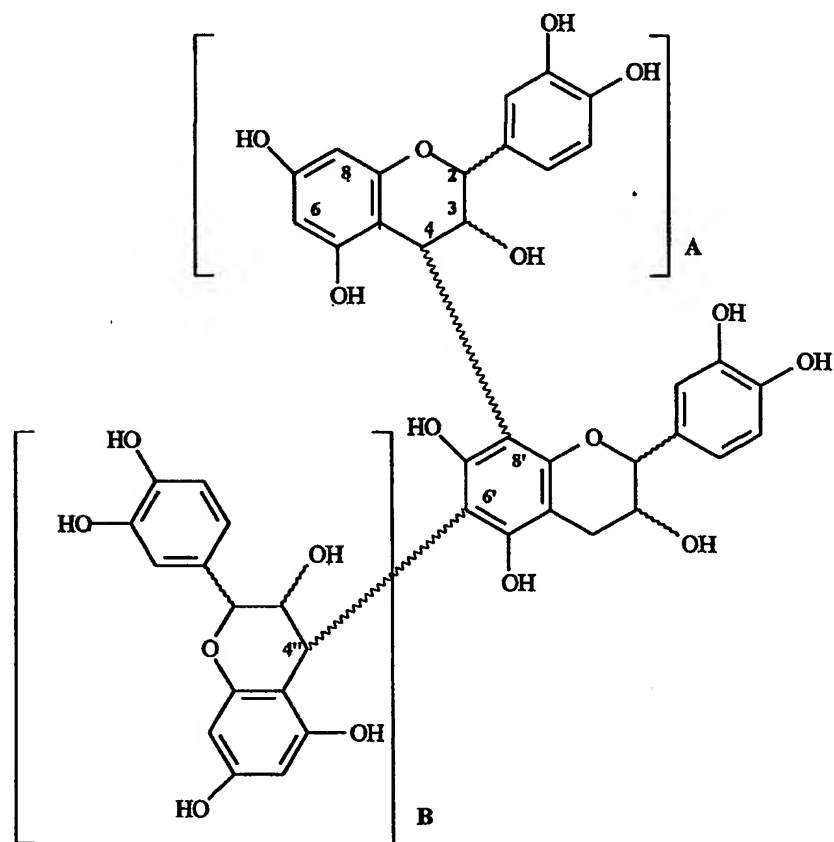


Linear oligomers where  $n$  is an integer from 0 to 16

5



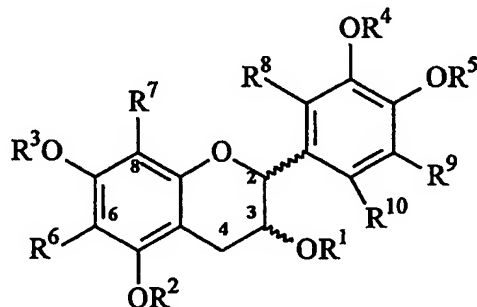
Branched oligomers where A and B are independently oligomers from 1 to 15 which total 3-18 in final oligomer,



In the oligomers  $n$  is an integer from 2 through 18, preferably 3 through 12, more preferably 5 through 12, and most preferably 5. The oligomers have interflavan linkages of (4→6) and and/or (4→8). The oligomers may be represented by the structures above. For the linear oligomer, when  $n$  is 0, the oligomer is termed a "dimer"; when  $n$  is 1 the oligomer is termed a "trimer"; when  $n$  is 2, the oligomer is termed a "tetramer"; when  $n$  is 3, the oligomer is termed a "pentamer"; and similar recitations may be designated for oligomers having  $n$  up to and including 16 and higher, such that when  $n$  is 16, the oligomer is termed an "octadecamer." For the branched oligomer, when  $A$  or  $B$  is 1, the oligomer is termed a "trimer"; with similar recitations such as those described for the linear oligomers.

Derivatives of the synthetic cocoa polyphenols include the gallated monomers and oligomers (a method for the preparation of the dimer di-gallate is disclosed in the International Appl. No. PCT/US00/08234, published as WO 00/61547 based on the U.S. Appl. Serial No. 09/289,565 filed April 9, 1999, the disclosure of which is incorporated by reference), the glycosylated monomers and oligomers, and mixtures thereof. Also included are metabolites of the monomers and oligomers, including the sulphated, glucuronidated, and methylated forms. Further included are the enzyme cleavage products generated by colonic microflora metabolism or internal mammalian metabolism.

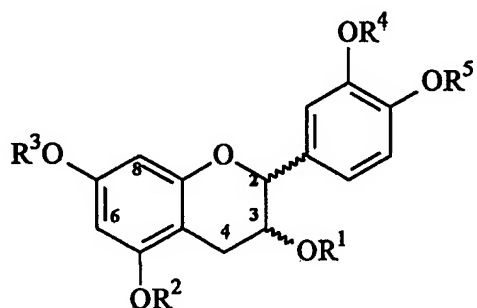
The methylated procyanidin monomer or oligomer is of formula  $(A)_n$  wherein  $n$  is 1 to 18 and  $A$  is a monomer unit of formula:



wherein each of  $R^1$  to  $R^5$ , and  $R^8$  to  $R^{10}$ , which are the same or different, is H or  $CH_3$ ; and  $R^6$  and  $R^7$ , which are the same or different, are H,  $CH_3$ , or a link to an adjacent monomer unit; provided that at least one of groups  $R^1$  to  $R^{10}$  in at least one monomer unit is  $CH_3$ . The monomers may, for example, be linked via any one or two of ring positions 4, 6, and 8 by interflavan linkages described above.

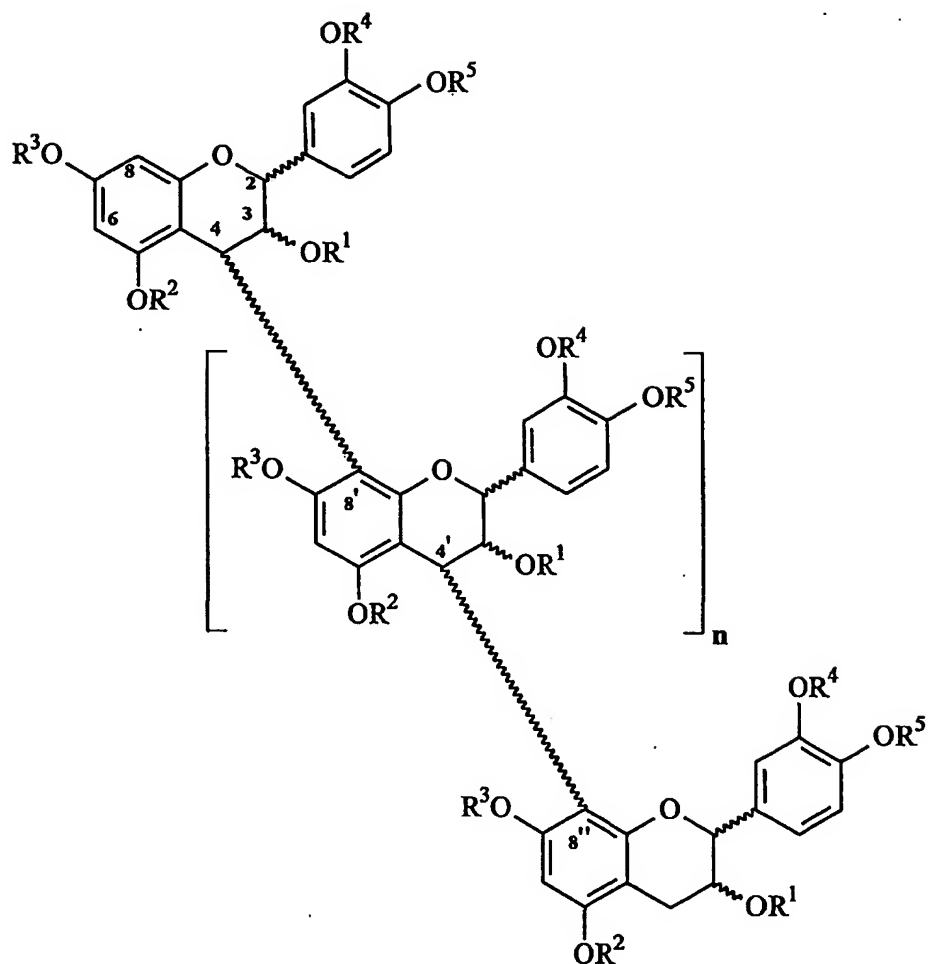
In another aspect of the invention, the methylated procyanidin monomer or oligomer is of formula  $(A')_n$  wherein  $n$  is 1 to 18 and  $A'$  is a monomer unit of formula:

10



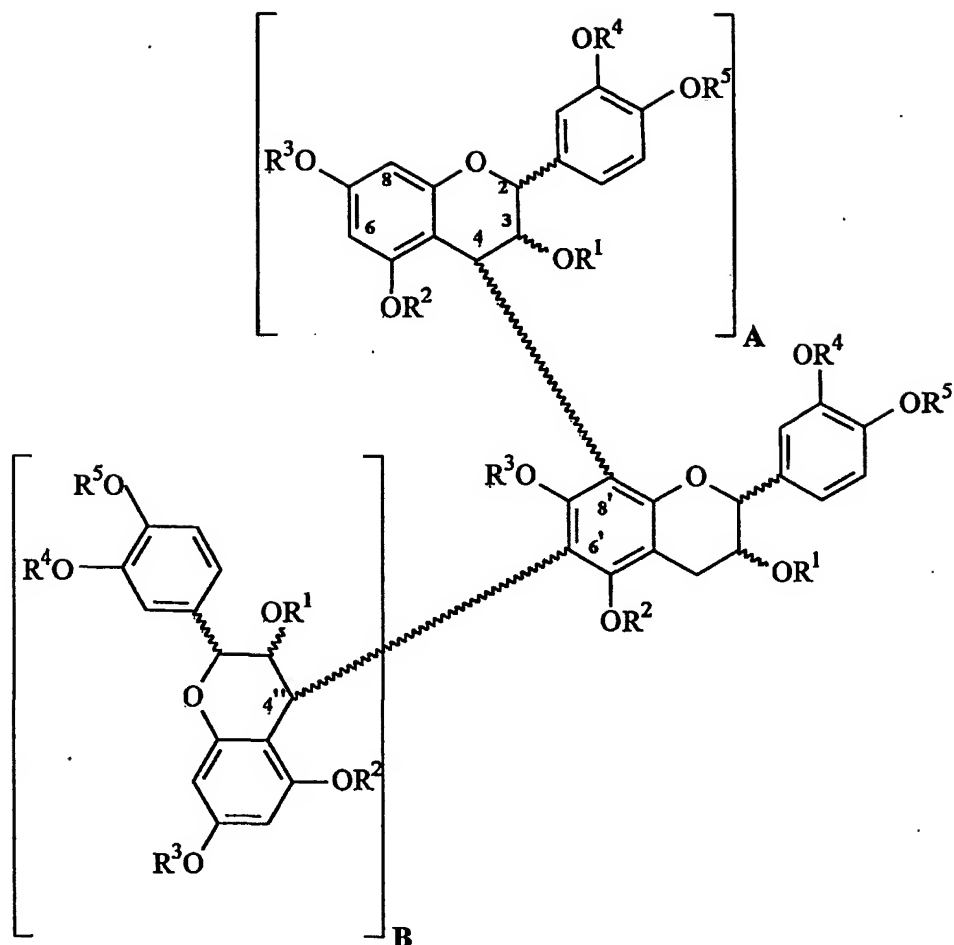
wherein each of  $R^1$  to  $R^5$ , which are the same or different, is H or  $CH_3$ , provided that at least one of groups  $R^1$  to  $R^5$  in at least one monomer unit is  $CH_3$ . The monomers may, for example, be linked via any one or two of ring positions 4, 6, and 8 by interflavan linkages described above.

For example, the oligomer may be a linear oligomer of the following structure wherein n is from 0 to 16:



5 wherein  $R^1$  to  $R^5$  is as defined above.

Alternatively, or in addition, the oligomer may be a branched oligomer of the following structure wherein A and B are independently oligomers from 1 to 15 which total 3-18 in final oligomer:



wherein  $R^1$  to  $R^5$  is as defined above.

5

In another embodiment, the invention provides a procyanidin oligomer or mixture of oligomers, wherein said oligomer is a methylated tetramer.

Monomers and/or oligomers of procyanidins obtained from tomato, peanuts including skins thereof, almonds including skins thereof, apple, cranberry and  
 10 blueberry may also be used to achieve beneficial effects described herein. Thus, any disclosure herein that relates to the use of cocoa procyanidins, or compositions containing cocoa procyanidins, also applies to the compounds from the above-enumerated sources.

The cocoa extracts are generally prepared by reducing cocoa beans to cocoa powder, defatting the powder, extracting the cocoa polyphenols, and purifying the extract. The cocoa powder can be prepared by freeze-drying the cocoa beans and pulp, depulping and dehulling the freeze-dried cocoa beans, and grinding the dehulled  
5 beans. The cocoa polyphenols can be extracted from the powder by solvent extraction techniques. The cocoa extracts can be purified, e.g., to be substantially pure, for instance, by gel permeation chromatography or by preparative High Performance Liquid Chromatography (HPLC) techniques or by a combination of such techniques.

With reference to the extraction and purification of the cocoa extracts, it will  
10 be understood that any species of *Theobroma*, *Herrania* or inter- and intra-species crosses thereof may be employed. In this regard, reference is made to Schultes, Synopsis of *Herrania*, "Journal of the Arnold Arboretum, Vol. XXXIX, pp 217 to 278, plus plates I to XVII (1985), Cuatrecasas, "Cocoa and Its Allies, A Taxonomic Revision of the Genus *Theobroma*," Bulletin of the United States National Museum,  
15 Vol. 35, page 6, pp. 379 to 613, plus plates 1 to 11 (Smithsonian Institution, 1964), and Addison, *et al.*, 'Observations on the Species of the Genus *Theobroma* Which Occurs in the Amazon," Bol. Tehc. Inst. Agronomico de Nortes, 25(3)(1951). The cocoa procyanidins can be isolated from cocoa or from any species within the *Theobroma* and *Herrania* genera. An outline of the purification protocol utilized in  
20 the isolation of substantially pure cocoa procyanidins is shown in Fig. 1. The steps of the purification process are outlined in Examples 1-5. The skilled artisan would appreciate and envision modification in the purification scheme outlined in Fig. 1 to obtain the active compounds without departing from the spirit or scope thereof and without undue experimentation.

25 Methylated derivatives of procyanidins may be prepared by reacting a procyanidin with a methylating agent. The procyanidin used in the reaction may be of natural origin, i.e., it may be extracted from cocoa beans as described, for example, in Examples 1-5, or may be prepared synthetically. The procyanidin to be used in the reaction may be a cocoa extract containing procyanidins, a fraction thereof containing  
30 at least one monomer or oligomer, or pooled fractions thereof. The fractions may be obtained, for example, as described herein. Thus, in one embodiment, the invention

relates to a method of methylating an isolated fraction of a cocoa extract which contains procyanidin oligomers such as tetramers.

A person of skill in the art will recognize suitable methylating agents. For example, anhydride, succinimide and halogenated methylating agents may be used. A  
5 diazomethane reagent may be used. The reaction conditions to be used for methylation will be recognized by persons of skill in the art and will depend on the methylating agent used and/or on the particular procyanidin being methylated. For example, when diazomethane reagent is used, a procyanidin may be combined with diazomethane at room temperature for a period of time suitable for the methylation to  
10 occur. In a preferred embodiment, the procyanidin is reacted with diazomethane for twenty minutes at room temperature and left to react overnight in the freezer; the reaction is stopped by using acetic acid in methanol; the solvent is removed and the solids are dried under vacuum. The reaction may be monitored using mass spectrometry. A person of skill in the art can optimize the conditions to obtain the  
15 desired degree of methylation. The procyanidin may be methylated at one or more hydroxyl groups, one or more carbon positions that are not sterically hindered, or both. In one embodiment a methylated procyanidin tetramer prepared, for example, using a diazomethane may be methylated at 14-18 of the OH groups. Some of the tetramer may be completely methylated at all 20 OH groups.

20 Monomers and/or oligomers of procyanidins may be obtained from tomato, peanuts including skins thereof, almonds including skins thereof, apple, cranberry and blueberry using the methods described herein and other known in the art, such as for example, as described in the International Appl. No. PCT/US99/05545 published as WO 99/45797; Hammerstone *et al.*, (2000) "Procyanidin Content and Variation in  
25 Some Commonly Consumed Foods," J. Nutr. Vol. 30 (suppl) 2086s-2092s; Lazarus *et al.*, (1999) "High Performance Liquid Chromatography/Mass Spectrophotometry Analysis of Proanthocyanidins in Foods and Beverages," J. Agr and Food Chem Vol. 37, No. 9, pp3693-3701 (relevant portions of which are hereby incorporated herein by reference); and publications cited therein. By way of example, peanut and almond  
30 procyanidins may be isolated as described below:



Raw peanuts are provided by M&M/MARS (Hackettstown, NJ). Raw almonds are provided by the Almond Board of California (Modesto, CA). The standards used are (-)-epicatechin and (+)-catechin. (Sigma Chemical, St. Louis, MO). Solid phase extraction (SPE) columns, (Supelcosil Envi-18 20 mL columns from  
5 Supelco, Inc., Bellefonte, PA) are rinsed with 3 x 5 mL of methanol and then conditioned with 3 x 5 mL of water prior to sample loading. After the appropriate sample loading and rinse procedures, the columns are dried under vacuum for 1-2 min. The SPE column is then soaked in 10 mL of acetone, water and acetic acid in a ratio by volume of 70:29.5:0.5, respectively, for 1 minute before the procyanidins are  
10 eluted off the column.

To extract the procyanidins from peanut skins, approximately 3.5 g of peanut skins are ground in a laboratory mill before being extracted in 25 mL of acetone, water and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. The suspension is centrifuged for 10 minutes at 1500 x g and the supernatant decanted.  
15 Twenty milliliters of water is added to the supernatant before the organic solvent is removed by rotary evaporation under partial vacuum at 45° C to yield approximately 22 mL of aqueous extract. The aqueous extract (22 mL) is loaded onto the preconditioned SPE column and rinsed with 40 mL of water. Then the procyanidins are eluted and collected. To extract the procyanidins from peanut nutmeat, the  
20 nutmeat is frozen in liquid nitrogen and then ground into a powder in a laboratory mill. The nutmeat powder (~10 g) is extracted three times with 45 mL of hexane to remove lipids. One gram of the resultant defatted nutmeat is extracted with 5 mL of acetone, water and acetic acid in a ratio by volume of 70:29.5:0.5, respectively.

With the almond seedcoat, approximately 24 g of seedcoat are removed from  
25 the raw almonds using a razor blade. The seedcoat is then defatted twice with 135 mL of hexane and centrifuged for 10 minutes at 1500 x g to yield approximately 14.6 g of defatted material. The defatted seedcoat is extracted with 90 mL of acetone, water and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. Thirty milliliters of water are added to the supernatant and the resulting acidified aqueous  
30 acetone is rotary evaporated under partial vacuum at 45° C to a final volume of 50 mL. The aqueous solution is loaded onto the preconditioned SPE column, rinsed with approximately 10 mL of water and the procyanidins eluted and collected.

The cocoa extracts and/or fractions having activity, without wishing to necessarily be bound by any particular theory, have been identified as cocoa polyphenol(s), which include procyanidins. These cocoa procyanidins have the effect as non-steroidal antiinflammatory agents, as modulators of the immune system, and as  
5 modulators of cytokine levels in white blood cells (monocytes, lymphocytes, macrophages etc.).

It has been surprisingly found that the cocoa procyanidins have discrete activities, and as such, the cocoa procyanidins have broad applicability to the treatment of a variety of disease conditions. The cocoa procyanidins can be used to  
10 modulate the immune system, to modulate the inflammatory response, and to modulate cytokine levels in white blood cells (monocytes, lymphocytes, macrophages etc.).

Thus, in one aspect, the invention relates to the use of the compound(s) described herein for the manufacture of a medicament, food or dietary supplement for  
15 treating asthma or for improving the respiratory tract pathology associated with asthma. Monomers and/or oligomers of cocoa procyanidins suitable for this use may be in a form of a crude or purified cocoa extract containing cocoa procyanidins. The cocoa extract may be prepared by the step of removing theobromine and/or caffeine from a crude procyanidin containing cocoa extract. Individual and pooled fractions  
20 containing at least one procyanidin may also be used. In one embodiment, a cocoa extract containing oligomeric fractions, e.g. at least 50 % by weight oligomers, may be used. In another embodiment, a composition comprising higher oligomeric fractions, e.g., trimer through decamer is used. Individual higher oligomers, e.g. trimer, tetramer, pentamer, hexamer, heptamer, octamer, nonamer, and decamer, or a  
25 lower oligomer, a dimer, may be used. Also suitable are synthetically prepared counterparts and cocoa procyanidin derivatives including methylated metabolites. Cocoa procyanidins may also be used in a form of a product processed in a way to preserve the levels of cocoa procyanidins, for example, high cocoa polyphenol solids. The medicament, food or dietary supplement comprises an amount of cocoa  
30 procyanidins that is effective to achieve the above asthma applications. A person of skill in the art will understand that the amount of cocoa procyanidin per unit of medicament, food, or dietary supplement will depend on the intended number of

administrations per day. That amount may be determined by routine experimentation using the experimental results and dosages disclosed herein as a guide.

In one of the preferred embodiments, a method for treating asthma or improving a respiratory tract pathology associated with asthma by administering the compound(s) of the invention to a mammal suffering from asthma, such as a human or a veterinary animal, is provided. The veterinary animal may be from a feline, canine or equine family, most typically a cat, a dog or a horse.

As a person of skill in the art will understand, a treatment requires administration of the cocoa procyanidins according to a regimen, *i.e.*, not sporadically. The administration may be, for example, on a daily basis for a predetermined period of time. A person of skill in the art may determine the frequency and duration of the administration using as a guide the disclosure herein and the general knowledge in the art. For example, treatment may be continued without interruption on a daily basis for at least a period of several weeks (e.g. two weeks), a month, or several months (e.g. two months, three months). The treatment may be repeated as needed and as determined by a person of skill in the art.

The administration dose may vary according to the type of the administration used, e.g. oral versus nasal. However, providing about 1-2  $\mu\text{M}$  concentration of cocoa procyanidins, for example, to the respiratory tract, gastro-intestinal tract or plasma would cause the desired beneficial effect. A suitable concentration to be achieved by administration may be from about 0.1 to about 20  $\mu\text{M}$ , more preferably from about 1 to about 10  $\mu\text{M}$ , and most preferably from about 1 to about 5  $\mu\text{M}$ . The concentration may be maintained, for example, for a period of about ten hours, about four hours, or about one hour.

By way of illustration, cocoa procyanidin delivered orally may be administered at from about 50 mg/day to about 1000 mg/day, preferably from about 100-150 mg/day to about 900 mg/day, and most preferably from about 300 mg/day to about 500 mg/day. Similar dosages may be used for nasal administration, however, lower dosages would also be effective given the topical nature of administration. Thus, less than 50 mg/day, e.g. about 20 to about 40 mg is also suitable for nasal administration. The amount of procyanidins may be determined by the methods described in Adamson et al. ("HPLC Method for the Quantification of Procyanidins in

Cocoa and Chocolate Samples and Correlation to Total Antioxidant Capacity" J. Ag. Food Chem., Vol. 7:10, 4184-4188), the relevant portion of which is hereby incorporated herein by reference.

In another aspect, the invention relates to the use of the compound(s)  
5 described herein for the manufacture of a medicament, food or dietary supplement for preventing a viral infection, or for reducing the risk of a viral infection, in a subject exposed to a virus. The subject is a mammal such as a human or a veterinary animal, which is exposed to an environment containing infective viral particles. Monomers and/or oligomers of cocoa procyanidins suitable for this use may be in a form of a  
10 crude or purified cocoa extract containing cocoa procyanidins. The cocoa extract may be prepared by the step of removing theobromine and/or caffeine from a crude procyanidin containing cocoa extract. Individual and pooled fractions containing at least one procyanidin may also be used. In one embodiment, a cocoa extract containing oligomeric fractions, e.g. at least 50 % by weight oligomers, may be used.  
15 In another embodiment, a composition comprising higher oligomeric fractions, e.g., trimer through decamer is used. Individual higher oligomers, e.g. trimer, tetramer, pentamer, hexamer, heptamer, octamer, nonamer, and decamer, or a lower oligomer, a dimer may be used. Also suitable are synthetically prepared counterparts thereof and cocoa procyanidin derivatives including methylated metabolites. Cocoa procyanidins  
20 may also be used in a form of a product processed in a way to preserve the levels of cocoa procyanidins, for example, high cocoa polyphenol solids. The medicament, food or dietary supplement comprises an amount of cocoa procyanidins that is effective to achieve the above anti-viral applications. A person of skill in the art will understand that the amount of cocoa procyanidin per unit of medicament, food, or  
25 dietary supplement will depend on the intended number of administrations per day. That amount may be determined by routine experimentation using the experimental results and dosages disclosed in the application as a guide.

Thus, a method is provided for preventing a viral infection, or for reducing the risk of a viral infection, in a mammal by administering to the mammal the  
30 compound(s) of the invention. In certain embodiments, infections of the nasal tract caused by a rhinovirus or any serotype of the myxovirus are prevented or attenuated.

The cocoa polyphenols may be administered in dosages and to achieve body concentrations as described above for asthma applications.

In yet another aspect, the invention relates to the use of the compound(s) described herein for the manufacture of a medicament, food or dietary supplement for  
5 treating a viral infection, or for reducing the symptoms of a viral infection, in a subject infected with a virus. The subject is a mammal such as a human or a veterinary animal, which has been infected with a virus. Thus, the invention relates to a method for treating a viral infection, or for reducing symptoms of a viral infection, in a mammal by administering to the mammal an effective amount of the  
10 compound(s) of the invention. In certain embodiments, infections of the nasal tract caused by a rhinovirus or any serotype of the myxovirus are prevented or attenuated. The cocoa polyphenols may be administered in dosages and to achieve body concentrations as described above for asthma applications.

Asthma, viral infections and other inflammatory disease disclosed herein are  
15 affected by the presence of cytokines. The effect of cocoa procyanidins and cocoa procyanidin extract on cytokine production in peripheral blood monocytes *in vitro*, is described herein. Selected procyanidin oligomers were shown to modulate the expression of at least five cytokines known to be crucially involved in the development of an immune response and in the inflammatory pathway. Interestingly,  
20 it appears that the modulatory effect of the individual procyanidin fractions on various cytokines differs, *i.e.*, some have an inhibitory effect whilst others have an enhancing effect on cell cytokine production *in vitro*. This raises the possibility that the biological activity of the individual cocoa procyanidin oligomers depends upon molecular size and shape. However, a person of skill in the art may determine the  
25 activity of each procyanidin using the experimental models described herein and may design cocoa procyanidin uses accordingly. Therefore, the cocoa procyanidin oligomers also have a utility in modulating an immune response as part of a therapeutic regime to treat human diseases which are caused by inappropriate activation or repression of the immune system.

30 The following cytokines were studied with respect to their gene expression by, and secretion from human peripheral blood mononuclear cells (PBMCs) and the effect of cocoa procyanidins thereon is reported in Examples 7 and 8.

Interleukin 1 (IL-1) is described as the prototypic cytokine which, unlike other lymphocyte growth factors, is multifunctional and exerts its effect on a wide variety of cell types (Dinarello, "Interleukin-1, Interleukin-1 Receptors And Interleukin-1 Receptor Agonists", Intern. Rev. Immunol. 16:457-499 (1998)). Some activities include: activation of T cells, induction of acute phase protein synthesis by hepatocytes, and induction of fever. IL-1 is also reported to stimulate hematopoiesis by inducing the proliferation of pluripotent progenitors in the bone marrow (Huggins *et al.*, "IL-1 Induction Of Cyclooxygenase-2 Expression In Rat Mesangial Cells", Pharmac. Ther. 59: 55-123, 1993). IL-1 is one of the most important inducers of transcription of the iNOS (inducible nitric oxide synthase) gene. iNOS activity results in the production of nitric oxide by macrophages.

Interleukin 2 (IL-2) is a T-cell growth factor present in tissue culture supernatants. IL-2 has been reported to participate in the activation and growth of NK cells, augment interferon- $\gamma$  production, and induce IL-6 production by human monocytes. Like IL-1, it is one of the crucial inducers of transcription of the iNOS gene.

Interleukin 4 (IL-4) is a pleiotropic cytokine possessing a broad spectrum of biological effects on several types of target cells. It is involved in the activation, proliferation and differentiation of B-cells. Although it is considered to be an anti-inflammatory cytokine, it is known to enhance the production of IgE, which is responsible for the generation of many of the symptoms of the allergic reaction. Additionally, IL-4 induces proliferation of T-cells and mast cells, activates and suppresses macrophages and inhibits proinflammatory cytokine production by Th1 cells.

Interleukin 6 (IL-6) is a pleiotropic cytokine produced by a wide variety of cells which has been reported to regulate immunological responses, hematopoiesis, and acute phase reactions. Other major activities include: weak antiviral activity, induction of cytotoxic T-cell differentiation; and growth inhibition of certain myeloma cell lines, myeloid leukemic cell lines, and breast carcinoma cell lines. Additionally, accumulating evidence supports an essential role of IL-6 in the development, differentiation, regeneration and degeneration of neurons in the peripheral and central nervous system.

Tumor necrosis factor alpha (TNF $\alpha$ ) is a cytokine which is produced by mast cells, macrophages and lymphocytes, and which mediates inflammation and cytotoxic reactions. It is very important in the early on-set of an immune response, recruiting leukocytes via release from the endothelium. Furthermore, TNF $\alpha$  also causes NK  
5 cells to release IFN- $\gamma$ , which causes macrophage activation and induces a state of antiviral resistance in uninfected tissue cells. It is also involved in triggering the pathway leading to the production of NO from mouse macrophages.

Cytokines function as the messengers of the immune system: they assist in regulating the development and actions of immune effector cells, and also have  
10 effector functions of their own. For this reason the manipulation of cytokine levels can be seen as a potential source of disease therapy, particularly for conditions in which the pathology is caused largely by an inappropriate immune response. Furthermore, modulating cytokine levels could be developed into a prophylactic therapy for ill-health of all kinds: firstly by the enhancement of immune responses  
15 against external pathogens such as viruses and also cancer cells, and secondly by enhancing cytokines which have a stimulatory effect on the production of substances which are beneficial for the health, for example IL-1 and IL-2, which are both known to enhance NO levels in humans via the induction of the iNOS gene.

The cytokines studied here are known for their roles in the early development  
20 of an immune response against pathogens such as viruses and bacteria. Prior to the involvement of T-lymphocytes, TNF $\alpha$  and IL-1 recruit leukocytes to the endothelial surface and enable their cellular entry. They are responsible for activating macrophages and neutrophils, and for the production of IFN $\gamma$  by NK cells, which augments microbicidal activity. IL-1, IL-2 and IL-6 are all known to be involved in  
25 T-cell proliferation and activation, leading to further cytokine production and the activation of antigen presenting cells such as macrophages. Activated T-cells produce cytokines which are responsible for B-cell activation, population expansion and differentiation: IL-4 acts early on in B-cell proliferation; IL-2 causes B-cell proliferation; and IL-6 is a strong signal for B-cell differentiation into antibody  
30 forming cells. Cytotoxic T-cells are one of the cell-types involved in the defense against intracellular pathogens such as bacteria and viruses. The second major arm of

defense is the production of antibody, which recognizes antigen on the surface of an infected cell and recruits effector cells which kill the infected cell. Thus, the activity of the cytokines studied result in the production and activation of immune cells which make up the first line of defense against in-coming intracellular pathogens. Thus, the  
5 cocoa polyphenols, which modulate these cytokines, may be used to enhance and optimize the immune response, such that any in-coming pathogens, for example the viruses which cause the common cold (rhinovirus) and influenza (myxovirus), would be eliminated before they manifested their symptoms.

Due to their powerful effects on the human immune response, all of the  
10 cytokines studied here have been investigated for their potential in the treatment of human disease.

IL-1 has been used in the treatment of solid tumors, and can be given to patients as a co-treatment with marrow-suppressing chemotherapy, in order to decrease the nadir and duration of the marrow suppression (Dinarello, "IL-1, IL-1  
15 Receptors and IL-1 Receptor Antagonists" Intern. Rev. Immunol. 16:457-499, 1998). Current research is investigating the role of IL-1 and other inflammatory mediators in the biology of major depression: several lines of evidence indicate that IL-1 $\beta$  may have a role in the biology of major depression, and it may additionally be involved in the pathophysiology and somatic consequences of depression as well as in the effects  
20 of anti-depressant treatment (Licinio *et al.*, "The Role of Inflammatory Mediators in the Biology of Major Depression: CNS cytokines modulate the biological substrate of depressive symptoms, regulate stress-responsive systems, and contribute to neurotoxicity and neuroprotection" Mol. Psychiatry 4(4):317-327, 1999).

The use of immunostimulatory cytokines has become an increasingly  
25 promising approach in cancer immunotherapy. Inhaled IL-2 has been shown to prevent progress of pulmonary metastases effectively in 70% of patients (Huland *et al.*, "Treatment of Pulmonary Metastatic Renal-cell Carcinoma in 116 patients Using Inhaled IL-2" Anticancer Res. 19(4a):2679-83, 1999). It has been shown that cells genetically engineered to produce IL-2 and injected into a sick animal can be effective  
30 in eradicating disseminated tumors in mice (Mackensen *et al.*, "Immunostimulatory Cytokines in Somatic Cells and Gene Therapy of Cancer" Cytokine Growth Factor Rev. 8(2):119-28, 1997), increasing host anti-tumor responsiveness (Govaerts *et al.*,



"Retroviral-Mediated Transfer of Genes Encoding IL-2 and IL-12 into Fibroblasts Increases Host Antitumor Responsiveness", *Cancer Gene Ther.* 6(5):447-55, 1999) and exerting an anti-leukemic effect in mice (Tam *et al.*, "Antileukemic Effect of IL-2-transduced Murine Bone Marrow After Autologous Transplantation", *Biol. Blood Marrow Transplant*, 5(4):231-42, 1999).

Manipulation of IL-4 levels are being investigated for roles in the treatment of humans of allergy, rheumatoid arthritis (Gallagher *et al.*, "Juvenile Rheumatoid Arthritis, *Curr. Opin. Rheumatol.* 11(5):372-6, 1999), asthma (Pauwels *et al.*, "Cytokines and their Receptors as Therapeutic Targets in Asthma" *Clin. Exp. Allergy*, 28 Suppl. 3:1-5, 1998) and inflammatory immune disease (Rocken *et al.*, "IL-4-Induced Immune Deviation as Antigen-Specific Therapy for Inflammatory Autoimmune Disease", *Immunol Today* 17(5):225-31, 1996). The concept of asthma as a condition in which acute and chronic inflammatory changes in the airways play a fundamental role is well established. Furthermore, the role of formation and release of inflammatory mediators, such as leukotrienes, nitric oxide (NO), and pro-inflammatory cytokines as crucial elements of these inflammatory processes is now supported by abundant laboratory and clinical evidence.

Furthermore, studies of mice have shown that IL-4 may have a role in preventing the onset of insulin-dependent diabetes (Cameron *et al.*, "Cytokine- and Costimulation-mediated Therapy of IDDM", *Crit Rev Immunol* 17(5-6):537-44, 1997).

Therefore, cocoa polyphenols may be used to treat, improve the immune response and/or alleviate the symptoms associated with inflammatory conditions such as allergy, rheumatoid arthritis, and asthma.

IL-6 is used as a haemopoietic growth factor, and the down-regulation of IL-6 has been used in the treatment of inflammatory bowel disease (Rogler *et al.*, "Cytokines in Inflammatory Bowel Disease", *World J Surg* 22(4):382-9, 1998). Gene therapy studies on rats have shown that IL-6 plays a significant role in the induction of inflammatory skin diseases, which leads to the possibility of the down-regulation of IL-6 production as a therapy for this condition (Sawamura *et al.*, "Induction of Keratinocyte Proliferation and Lymphocytic Infiltration by in vivo introduction of the IL-6 gene into keratinocytes and Possibility of Keratinocyte gene therapy for

Inflammatory Skin Diseases Using IL-6 Mutant Genes", *J Immunol*, 161 (10):5633-9, 1998). And lastly, studies by Arruda *et al.* have implicated IL-6 as a potential neuromodulator/ neurotransmitter, and shown that it may be involved in the neural process which leads to neuropathic pain ("Increase of IL-6 mRNA in the Spinal Cord Following Peripheral Nerve Injury in the Rat: Potential Role of IL-6 in Neuropathic Pain", *Brain Res Mol Brain Res*, 62(2):228-35, 1998). This leads to the intriguing possibility of using cytokine modulation in long-term pain management.

TNF $\alpha$  is a potent inflammatory cytokine, and reducing levels of the cytokine (by treatment with an anti- TNF $\alpha$  monoclonal antibody) has been utilized in the successful treatment of inflammatory bowel disease (for review, see Sandborn *et al.*, Antitumor Necrosis Therapy for Inflammatory Bowel Disease: a Review of Agents, Pharmacology, Clinical Results, and Safety", *Inflamm. Bowel Dis.* 5(2), 119-33, 1999) and rheumatoid arthritis (Ohshima *et al.*, "Long-term Follow-up of the Changes in Circulating Cytokines, Soluble Cytokine Receptors, and White Blood Cell Subset Counts in Patients with RA after Monoclonal anti-TNF alpha Antibody Therapy", *J Clin Immunol*, 19(5):305-13, 1999). Additionally, it has long been utilized in anti-cancer clinical trials. The most encouraging results have been in the treatment of intraperitoneal ovarian tumors, but it is known to reduce many types of malignant ascites.

The experimental design is summarized in Figure 2. The changes in the expression of cytokine genes in response to a particular cocoa procyanidin application were measured using gene-specific semi-quantitative PCR. This method directly shows the effects that the cocoa procyanidins have on the synthesis of cytokine mRNA. However, although PCR is a well-controlled, simple, rapid, reproducible, and non-radioisotopic method for analysis of cytokine mRNA, it may not be sufficient to obtain a complete understanding of the cytokine expression and secretion. It is known that control of cytokine protein synthesis and secretion is extremely complex, and can be regulated at many different levels apart from the transcriptional level. For example, the half life of the mRNA can be modified; the primary cDNA transcripts can fail to mature (for example, not achieving polyadenylation or undergoing alternative splicing to yield a non-functional cDNA); the cDNA may require additional modification to become stable enough for translation; the polypeptide itself requires extensive

modification before it assumes the tertiary structure of the native protein; and finally, the protein may require additional processing before it can be secreted from the cell where it was synthesized. This complexity of processing is exemplified by IL-1 $\beta$ : firstly, not all transcribed IL-1 $\beta$  is translated into protein. Moreover, a reserve of IL-1 $\beta$  protein is maintained in the cell cytosol in the form of pro-IL-1 $\beta$ , so stimulation of the cell may lead to cleavage of this protein and release of the mature cytokine without affecting transcription (Dinarello *et al.*, "IL-1, IL-1 Receptors and IL-1 Receptor Antagonists", Intern Rev Immunol, 16:457-499, 1998). For this reason it was decided to perform assays for cytokine protein secretion, in parallel with the RT-PCR.

Cocoa procyanidin extract and oligomeric fractions thereof modulate levels of cytokine gene expression and protein secretion in PBMCs *in vitro*. Therefore the cocoa procyanidin extract and/or the purified mixture of monomers and oligomers can be used to treat pathological conditions which are affected by cytokines, such as asthma and conditions associated with viral infections, and to maintain mammalian health by optimizing cytokine levels in the body.

#### FORMULATIONS AND METHODS

Therefore, the cocoa procyanidins, including monomers and oligomers, combinations thereof and compositions containing them exhibit a wide array of activities and functions such as modulating the immune system, modulating cytokine levels in peripheral blood monocyte cells and including having beneficial effects on inflammatory conditions such as asthma or conditions associated with an exposure to viruses.

Formulations of the cocoa procyanidins, combinations thereof and compositions containing them can be prepared with standard techniques well known to those skilled in the pharmaceutical, food science, medical and veterinary arts, in the form of a liquid, suspension, tablet, capsule, injectable solution, nasal delivery dosage forms or suppository, for immediate or slow-release of the active compounds.

The carrier may also be a polymeric delayed release system. Synthetic polymers are particularly useful in the formulation of a composition having controlled release. An early example of this was the polymerization of methyl methacrylate into

spheres having diameters less than one micron to form so-called nano particles, reported by Kreuter, J., *Microcapsules and Nanoparticles in Medicine and Pharmacology*, M. Donbrow (Ed). CRC Press, p. 125-148.

A frequent choice of a carrier for pharmaceuticals and more recently for  
5 antigens is poly (d,l-lactide-co-glycolide) (PLGA). This is a biodegradable polyester that has a long history of medical use in erodable sutures, bone plates and other temporary prostheses where it has not exhibited any toxicity. A wide variety of pharmaceuticals have been formulated into PLGA microcapsules. A body of data has accumulated on the adaption of PLGA for controlled release, for example, as  
10 reviewed by Eldridge, J.H., *et al.* *Current Topics in Microbiology and Immunology*, 1989, 146:59-66. The entrapment in PLGA microspheres of 1 to 10 microns in diameter can be effective when administered orally. The PLGA microencapsulation process uses a phase separation of a water-in-oil emulsion. The cocoa procyanidins are prepared as an aqueous solution and the PLGA is dissolved in a suitable organic  
15 solvents such as methylene chloride and ethyl acetate. These two immiscible solutions are co-emulsified by high-speed stirring. A non-solvent for the polymer is then added, causing precipitation of the polymer around the aqueous droplets to form embryonic microcapsules. The microcapsules are collected, and stabilized with one of an assortment of agents including polyvinyl alcohol (PVA), alginates and methyl  
20 cellulose. The solvent is subsequently removed by either drying *in vacuo* or solvent extraction.

Additionally, with regard to the preparation of slow-release formulations, the disclosures of U.S. Patent Nos. 5,024,843, 5,091,190, 5,082,668, 4,612,008 and 4,327,725 are hereby incorporated herein by reference.

25 Additionally, selective processing coupled with the identification of cocoa genotypes of interest could be used to prepare Standard-of-Identity (SOI) and non-SOI chocolate products as vehicles to deliver the active compounds to a patient in need of treatment for the disease conditions described above including asthma and viral infections, as well as a means for the delivery of conserved levels of the cocoa  
30 procyanidins.

A method of producing cocoa butter and/or cocoa solids having conserved levels of cocoa polyphenols from cocoa beans uses a unique combination of

processing steps which does not require separate bean roasting or liquor milling equipment, allowing for the option of processing cocoa beans without exposure to severe thermal treatment for extended periods of time and/or the use of solvent extraction of fat. The benefit of this process lies in the enhanced conservation of polyphenols in contrast to that found in traditional cocoa processing, such that the ratio of the initial amount of polyphenol found in the unprocessed bean to that obtainable after processing is less than or equal to 2. Partially defatted cocoa solids having a high cocoa polyphenol (CP) content, including a high cocoa procyanidin content, can be obtained by processing the cocoa beans directly to cocoa solids without a bean or nib roasting step. This method conserves the cocoa polyphenols because it omits the traditional roasting step. The method consists essentially of the steps of: a) heating the cocoa beans to an internal bean temperature just sufficient to reduce the moisture content to about 3% by weight and to loosen the cocoa shell; b) winnowing the cocoa nibs from the cocoa shells; c) screw pressing the cocoa nibs; and d) recovering the cocoa butter and partially defatted cocoa solids which contain cocoa polyphenols including cocoa procyanidins. Optionally, the cocoa beans are cleaned prior to the heating step, e.g., in an air fluidized bed density separator. The winnowing can also be carried out in the air fluidized bed density separator. Preferably, the cocoa beans are heated to an internal bean temperature of about 100°C to about 110°C, more preferably less than about 105°C, typically using an infra red heating apparatus for about 3 to 4 minutes. If desired, the cocoa solids can be alkalized and/or milled to a cocoa powder.

The internal bean temperature (IBT) can be measured by filling an insulated container such as a thermos bottle with beans (approximately 80 – 100 beans). The insulated container is then appropriately sealed in order to maintain the temperature of the sample therein. A thermometer is inserted into the bean-filled insulated container and the temperature of the thermometer is equilibrated with respect to the beans in the thermos. The temperature reading is the IBT temperature of the beans. IBT can also be considered the equilibrium mass temperature of the beans.

Cocoa beans can be divided into four categories based on their color, which corresponds to the degree of fermentation: predominately brown (fully fermented), purple/brown, purple, and slaty (unfermented). Preferably, the cocoa solids are

prepared from underfermented cocoa beans which have a higher cocoa polyphenol content than fermented beans. Underfermented beans include slaty cocoa beans, purple cocoa beans, mixtures of slaty and purple cocoa beans, mixtures of purple and brown cocoa beans, or mixture of slaty, purple, and brown cocoa beans. More preferably, the cocoa beans are slaty and/or purple cocoa beans.

As discussed above, the cocoa polyphenol (CP) content, including the cocoa procyanidin content, of roasted cocoa nibs, chocolate liquor, and partially defatted or nonfat cocoa solids is higher when they are prepared from cocoa beans or blends thereof which are underfermented, *i.e.*, beans having a fermentation factor of 275 or less. The "fermentation factor" is determined using a grading system for characterizing the fermentation of the cocoa beans. Slaty is designated 1, purple is 2, purple/brown is 3, and brown is 4. The percentage of beans falling within each category is multiplied by the weighted number. Thus, the "fermentation factor" for a sample of 100% brown beans would be  $100 \times 4$  or 400, whereas for a 100% sample of purple beans it would be  $100 \times 2$  or 200. A sample of 50% slaty beans and 50% purple beans would have a fermentation factor of 150  $[(50 \times 1) + (50 \times 2)]$ .

The compositions suitable for uses described herein can be administered to a subject or patient in need of such administration in dosages and by techniques well known to those skilled in the medical, nutritional or veterinary arts taking into consideration the data herein, and such factors as the age, sex, weight, genetics and condition of the particular subject or patient, the route of administration, relative concentration of particular oligomers, and toxicity (e.g., LD<sub>50</sub>).

Suitable compositions of the invention for human or veterinary use include edible compositions for oral administration, such solid or liquid formulations, for example, capsules, tablets, pills and the like; chewable solid formulations, beverage formulations, or dried beverage formulations for reconstitution; liquid preparations for orifice administration, e.g., by oral, by nasal, by anal, by vaginal administration via suspensions, syrups or elixirs; and ingestible preparations for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration e.g., suspensions or emulsions. The above compositions may be chocolate flavored if the high cocoa polyphenol solids are used in the composition. However, if the cocoa extract is used in the composition, chocolate or other flavoring agents may be

included in the composition, particularly if the composition is an edible composition. The active ingredient in the compositions may complex with proteins and, when administered into the bloodstream, clotting may occur due to the precipitation of blood proteins. The skilled artisan should take this into account. In such  
5 compositions the active cocoa procyanidin may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, DMSO, ethanol, or the like. The cocoa extract or cocoa procyanidin fractions can be provided in lyophilized form for reconstituting for example, in an edible liquid or in isotonic aqueous, saline, glucose or DMSO buffer. In certain saline solutions, some  
10 precipitation has been observed. Precipitation may be employed as a means to isolate cocoa procyanidins, e.g. by a "salting out" procedure.

In one embodiment, a dosage form suitable for nasal delivery is provided. The dosage form may be prepared using the methods known in the art and the cocoa polyphenols described herein and may be, for example, an inhaler (e.g. metered dose  
15 inhaler), a nebulizer (e.g. aerosol nebulizer, ultrasonic nebulizer), nasal drops, or a nasal spray. In a preferred embodiment, the dosage form contains cocoa procyanidins in an amount effective to achieve asthma and anti-viral health benefits described in detail above.

An additional composition is a cocoa procyanidin-enriched beverage. A  
20 preferred beverage or beverage mix comprises: high cocoa polyphenol solids and/or cocoa extract and, optionally, a natural or artificial sweetener and/or a natural or synthetic flavorant. The sweetener may be a sugar syrup/solids, or a sugar substitute. The term "sugar substitute" includes bulking agents, sugar alcohols, (*i.e.*, polyols such as glycerol), high potency sweeteners or combinations thereof. Nutritive  
25 carbohydrate sweeteners with varying degrees of sweetness intensity may be any of those typically used in the art and include, but are not limited to, sucrose, dextrose, fructose, lactose, maltose, glucose syrup solids, corn syrup solids, invert sugar, hydrolyzed lactose, honey, maple sugar, brown sugar, molasses and the like. Sugar substitutes may partially or totally replace the nutritive carbohydrate sweetener. High  
30 potency sugar substitutes include aspartame, cyclamates, saccharin, acesulfame-K, neohesperidin, dihydrochalcone, sucralose, alitame, *stevia* sweeteners, glycyrrhizin, thaumatin and the like as well as mixtures thereof. Exemplary sugar alcohols include

those typically used in the art such as sorbitol, mannitol, xylitol, maltitol, isomalt, lactitol and the like.

A dietary supplement containing the compounds of the invention may be prepared to contain dietary supplement nutrients selected from the group consisting of  
5 dicalcium phosphate, magnesium stearate, calcium nitrate, vitamins and minerals.

Example 9 describes the formulation of cocoa procyanidins tablets, for use in the pharmaceutical, diet supplement and food areas. Example 10 describes the preparation of the cocoa procyanidins as capsules for similar applications. Example 11 describes the preparation of Standard of Identity (SOI) and non-SOI chocolates  
10 containing the high cocoa polyphenol extract or cocoa solids obtained from methods described herein.

#### KITS

The active cocoa extract may be provided in a kit that may include a separate container containing a suitable carrier, diluent or excipient, and optionally other active  
15 ingredients which will depend upon the health benefit to be achieved, and additional agent(s) which can be provided in separate container(s) or in admixture with the active cocoa procyanidin(s). The kit may also include instructions for mixing or combining the ingredients and/or the administration.

In one embodiment, the instructions for administration are combined in a  
20 package with a composition suitable for oral delivery, such as a food, dietary supplement or pharmaceutical. Thus, the package contains a composition of the invention in combination with a label instructing to use the composition for at least one of the health benefits disclosed herein, e.g. treating asthma, reducing respiratory tract pathology associated with asthma or a viral infection, preventing a viral  
25 infection, enhancing an immune response to a viral infection or treating a viral infection. For example, a chocolate package comprising a composition as described herein and a label reciting e.g. reduces the risk of viral infection, improves immune response to viral infection, treats asthma or improves pathology associated with asthma is provided.

30 In another embodiment, a package comprising a nasal delivery dosage form and a label or instructions for use for asthma or anti-viral applications described herein is also provided.



**EXAMPLES****Example 1****Cocoa Source and Method of Preparation**

Several *Theobroma cacao* genotypes which represent the three recognized horticultural races of cocoa (Enriquez *et al.*, Cocoa Cultivars Register IICA, Turrialba, Cost Rica 1967; Engels, Genetic Resources of Cacao: A Catalogue of the CATIE Collection, Tech. Bull. 7, Turrialba, Costa Rica, 1981) were obtained from the three major cocoa producing origins of the world. A list of those genotypes used in this study are shown in Table 1. Harvested cocoa pods were opened and the beans with pulp were removed for freeze drying. The pulp was manually removed from the freeze dried mass and the beans were subjected to analysis as follows. The unfermented, freeze dried cocoa beans were first manually dehulled, and ground to a fine powdery mass with a TEKMAR Mill. The resultant mass was then defatted overnight by Soxhlet extraction using redistilled hexane as the solvent. Residual solvent was removed from the defatted mass by vacuum at ambient temperature.

**Table 1: Description of *Theobroma cacao* Source Material**

GENOTYPE	ORIGIN	HORTICULTURAL RACE
UIT-1	Malaysia	Trinitario
Unknown	West Africa	Forastero
ICS-100	Brazil	Trinitario (Nicaraguan Criollo ancestor)
ICS-39	Brazil	Trinitario (Nicaraguan Criollo ancestor)
UF-613	Brazil	Trinitario
EEG-48	Brazil	Forastero
UF-12	Brazil	Trinitario
NA-33	Brazil	Forastero

## Example 2

### Procyanidin Extraction Procedures

#### A. Method 1

- 5 Procyanidins were extracted from the defatted, unfermented, freeze dried cocoa beans of Example 1 using a modification of the method described by Jalal and Collin ('Polyphenols of Mature Plant, Seedling and Tissue Cultures of *Theobroma Cacao*', Phytochemistry, 6, 1377-1380, 1977). Procyanidins were extracted from 50 gram batches of the defatted cocoa mass with 2X 400 mL 70% acetone/deionized
- 10 water followed by 400mL 70% methanol/deionized water. The extracts were pooled and the solvents removed by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was diluted to 1L with deionized water and extracted 2X with 400mL CHCl<sub>3</sub>. The solvent phase was discarded. The aqueous phase was then extracted 4X with 500mL ethyl acetate. Any resultant emulsions were
- 15 broken by centrifugation on a Sorvall RC 28S centrifuge operated at 2,000 x for 30 min. at 10°C. To the combined ethyl acetate extracts, 100-200mL deionized water was added. The solvent was removed by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was frozen in liquid N<sub>2</sub> followed by freeze drying on a LABCONCO Freeze Dry System. The yields of crude
- 20 procyanidins that were obtained from the different cocoa genotypes are listed in Table 2.

**Table 2: Crude Procyanidin Yields**

GENOTYPE	ORIGIN	YIELDS (g)
UIT-1	Malaysia	3.81
Unknown	West Africa	2.55
ICS-100	Brazil	3.42
ICS-39	Brazil	3.45
UF-613	Brazil	2.98
EEG-48	Brazil	3.15
UF-12	Brazil	1.21

NA-33	Brazil	2.23
-------	--------	------

**B. Method 2**

Alternatively, procyanidins are extracted from defatted, unfermented, freeze dried cocoa beans of Example 1 with 70% aqueous acetone. Ten grams of defatted material was slurried with 100 mL solvent for 5-10 min. The slurry was centrifuged  
5 for 15 min. at 4°C at 3000 x g and the supernatant passed through glass wool. The filtrate was subjected to distillation under partial vacuum and the resultant aqueous phase frozen in liquid N<sub>2</sub>, followed by freeze drying on a LABCONCO Freeze Dry System. The yields of crude procyanidins ranged from 15-20%.

Without wishing to be bound by any particular theory, it is believed that the  
10 differences in crude yields reflected variations encountered with different genotypes, geographical origin, horticultural race, and method of preparation.

**Example 3****Partial Purification of Cocoa Procyanidins by Gel Permeation chromatography****A. Method 1**

15 Procyanidins obtained from Example 2 were partially purified by liquid chromatography on Sephadex LH-20 (28 x 2.5 cm). Separations were aided by a step gradient from deionized water into methanol. The initial gradient composition started with 15% methanol in deionized water which was followed step wise every 30 min. with 25% methanol in deionized water, 35% methanol in deionized water, 70%  
20 methanol in deionized water, and finally 100% methanol. The effluent following the elution of the xanthine alkaloids (caffeine and theobromine) was collected as a single fraction. The fraction yielded a xanthine alkaloid free subfraction which was submitted to further subfractionation to yield five subfractions designated MM2A through MM2E. The solvent was removed from each subfraction by evaporation at  
25 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was frozen in liquid N<sub>2</sub> and freeze dried overnight on a LABCONCO Freeze Dry System. A representative gel permeation chromatogram showing the fractionation is shown in Figure 3. Approximately, 100 mg of material was subfractionated in this manner.

30 Chromatographic Conditions: Column; 28 x 2.5 cm Sephadex LH-20, Mobile

Phase: Methanol/Water Step Gradient, 15:85, 25:75, 35:65, 70:30, 100:0 Stepped at ½ Hour Intervals, Flow Rate; 1.5mL/min, Detector; UV at  $\lambda_1 = 254$  nm and  $\lambda_2 = 365$  nm, Chart Speed: 0.5mm/min, Column Load; 120mg.

#### B. Method 2

- 5        Procyanidins obtained as in Example 2 were partially purified by liquid chromatography on Sephadex LH 20 (72.5 x 2.5cm), using 100% methanol as the eluting solvent, at a flow rate of 3.5mL/min. Fractions of the eluent were collected after the first 1.5 hours, and the fractions were concentrated by a rotary evaporator, redissolved in water and freeze dried. These fractions were referred to as pentamer  
10 enriched fractions. Approximately 2.00g of the extract obtained from Example 2 was subfractionated in this manner.

### Example 4

#### Analytical HPLC Analysis of Procyanidin Extracts

##### Method 1. Reverse Phase Separation

- 15        Procyanidin extracts obtained from Examples 2 & 3 were filtered through a 0.45 $\mu$  filter and analyzed by a Hewlett Packard 1090 ternary HPLC system equipped with a Diode Array detector and a HP model 1046A Programmable Fluorescence Detector. Separations were effected at 45°C on a Hewlett-Packard 5 $\mu$  Hypersil ODS column (200 x 2.1mm). The flavanols and procyanidins were eluted with a linear  
20 gradient of 60% B into A followed by a column wash with B at a flow rate of 0.3mL/min. The mobile phase composition was B = 0.5% acetic acid in methanol and A = 0.5% acetic acid in nanopure water. Acetic acid levels in A and B mobile phases can be increased to 2%. Components were detected by fluorescence, where  $\lambda_{ex} = 276$ nm and  $\lambda_{ex} = 316$ nm and by UV at 280nm. Concentrations of (+)-catechin and (-)  
25 )-epicatechin were determined relative to reference standard solutions. Procyanidin levels were estimated by using the response factor for (-)-epicatechin. A representative HPLC chromatogram showing the separation of the various components is shown in Figure 4 for one cocoa genotype. Similar HPLC profiles were obtained from the other cocoa genotypes.
- 30 **HPLC Conditions:** Column: 200 x 2.1mm Hewlett Packard Hypersil ODS (5 $\mu$ )

Guard column: 20 x 2.1mm Hewlett Packard Hypersil ODS  
(5 $\mu$ )

Detectors: Diode Array @ 280nm  
Fluorescence  $\lambda_{ex}$  = 276nm;  
5  $\lambda_{em}$  = 316nm.

Flow rate: 0.3mL/min.

Column Temperature: 45°C

Gradient: Time (min)	0.5% Acetic Acid in nanopure water	0.5% Acetic acid in methanol
0	100	0
50	40	60
60	0	100

#### **Method 2. Normal Phase Separation**

- Procyanidin extracts obtained from previous examples were filtered through a  
10 0.45 $\mu$  filter and analyzed by a Hewlett Packard 1090 Series II HPLC system equipped  
with a HP model 1046A Programmable Fluorescence detector and Diode Array  
detector. Separations were effected at 37°C on a 5 $\mu$  Phenomenex Lichrosphere®  
Silica 100 column (250 x 3.2mm) connected to a Supelco Supelguard LC-Si 5 $\mu$  guard  
column (20 x 4.6mm). Procyanidins were eluted by linear gradient under the  
15 following conditions: (Time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65,  
10, 86), (70, 10, 86) followed by an 8 min. re-equilibration. Mobile phase  
composition was A=dichloromethane, B=methanol, and C=acetic acid: water at a  
volume ratio of 1:1. A flow rate of 0.5 mL/min. was used. Components were  
detected by fluorescence, where  $\lambda_{ex}$  = 276nm and  $\lambda_{em}$  = 316nm or by UV at 280 nm.  
20 A representative HPLC chromatogram showing the separation of the various  
procyanidins is shown in Figure 5 for one genotype. Similar HPLC profiles were  
obtained from other cocoa genotypes.

**HPLC Conditions:** 250 x 3.2mm Phenomenex Lichrosphere® Silica 100 column  
(5 $\mu$ ) 20 x 4.6mm Supelco Supelguard LC-Si (5 $\mu$ ) guard  
25 column

Detectors: Photodiode Array @ 280nm  
Fluorescence  $\lambda_{ex}$  = 276nm;

$$\lambda_{\text{em}} = 316\text{nm.}$$

Flow rate: 0.5 mL/min.

Column Temperature: 37°C

Gradient: Time (min.)	CH <sub>2</sub> -Cl <sub>2</sub>	Methanol	Acetic Acid/Water (1:1)
0	82	14	4
30	67.6	28.4	4
60	46	50	4
65	10	86	4
70	10	86	4

5

### Example 5

#### Purification of Oligomeric Fractions from Pentamer Enriched Fractions

##### Preparative Normal Phase Separation

The pentamer enriched fractions obtained as in Example 4 were further purified by preparative normal phase chromatography by modifying the method of

10 Rigaud *et al.*, (*J. Chrom.* 654: 255-260, 1993).

Separations were affected at ambient temperature on a 5 $\mu$  Supelcosil LC-Si 100Å column (50 x 2cm), with an appropriate guard column. Procyanidins were eluted by a linear gradient under the following conditions: (time, %A, %B, flow rate); (0, 92.5, 7.5, 10); (10, 92.5, 7.5, 40); (30, 91.5, 18.5, 40); (145, 88, 22, 40);  
 15 (150, 24, 86, 40); (155, 24, 86, 50); (180, 0, 100, 50). Prior to use, the mobile phase components were mixed by the following protocol:

Solvent A preparation (82% CH<sub>2</sub>Cl<sub>2</sub>, 14% methanol, 2% acetic acid, 2% water):

1. Measure 80 mL of water and dispense into a 4L bottle.
- 20 2. Measure 80 mL of acetic acid and dispense into the same 4L bottle.
3. Measure 560 mL of methanol and dispense into the same 4L bottle.
4. Measure 3280 mL of methylene chloride and dispense into the 4L bottle.
5. Cap the bottle and mix well.

6. Purge the mixture with high purity Helium for 5-10 minutes to degas.

Repeat steps 1-6 two times to yield 8 volumes of solvent A.

Solvent B preparation (96% methanol, 2% acetic acid, 2% water):

1. Measure 80 mL of water and dispense into a 4L bottle.
2. Measure 80 mL of acetic acid and dispense into the same 4L bottle.
3. Measure 3840 mL of methanol and dispense 3840 mL of methanol and dispense into the same 4L bottle.
4. Cap the bottle and mix well.
5. Purge the mixture with high purity Helium for 5-10 minutes to degas.

10

Repeat steps 1-5 to yield 4 volumes of solvent B. Mobile phase composition was A = methylene chloride with 2% acetic acid and 2% water; B = methanol with 2% acetic acid and 2% water. The column load was 0.7g in 7mL. components were detected by UV at 254nm. A typical preparative normal phase HPLC separation of cocoa procyanidins is shown in Figure 5.

15

**HPLC Conditions:** Column: 50 x 2cm 5 $\mu$  Supelcosil LC-Si run @ ambient temperature.

Mobile Phase: A = Methylene Chloride with 2%  
Acetic Acid and 2% Water.

20

B = Methanol with 2% Acetic Acid and 2% Water.

Gradient/Flow Profile:

TIME (MIN)	%A	%B	FLOW RATE (mL/min)
0	92.5	7.5	10
10	92.5	7.5	40
30	91.5	8.5	40
145	88.0	22.0	40
150	24.0	86.0	40
155	24.0	86.0	50
180	0.0	100.0	50

### Example 6

#### MALDI - TOF/MS Analysis of

##### High Molecular Weight Procyanidin Oligomers

An analytical examination was made on GPC eluants associated with high  
5 molecular weight procyanidin oligomers as prepared in Example 3, Method A. The  
objective was to determine whether procyanidin oligomers with  $n > 18$  were present.  
If present, these oligomers represent additional cocoa procyanidins. Adjustments to  
existing methods of isolation, separation and purification embodied in the invention  
can be made to obtain these oligomers for subsequent *in vitro* and *in vivo* evaluation  
10 for anti-cancer, anti-tumor or antineoplastic activity, antioxidant activity, inhibit DNA  
topoisomerase II enzyme, inhibit oxidative damage to DNA, and have antimicrobial,  
NO or NO-synthase, apoptosis, platelet aggregation, and blood or *in vivo* glucose-  
modulating activities, as well as efficacy as non-steroidal anti-inflammatory agents.

Figure 6 represents a MALDI-TOF mass spectrum of the GPC eluant sample  
15 described above. The  $[M + Na]^+$  and/or  $[M + K]^+$  and/or  $[M + 2Na]^+$  ions  
characterizing procyanidin oligomers representative of tetramers through  
octadecamers are clearly evident.

It was learned that an acid and heat treatment will cause the hydrolysis of  
procyanidin oligomers. Therefore, the invention comprehends the controlled  
20 hydrolysis of high molecular weight procyanidin oligomers (e.g. where  $n$  is 13 to 18)  
as a method to prepare lower molecular weight procyanidin oligomers (e.g. where  $n$  is  
2 to 12).

### Example 7

#### The Effect of Cocoa Procyanidins on Cytokine Gene Expression *in vitro*

25 The following experiments were concerned with ascertaining whether or not  
the cocoa procyanidins modulate the production of cytokines, which are known to be  
involved in the inflammatory/immune responses by human peripheral blood  
mononuclear cells (PBMC's).

To ascertain the differential effects of the cocoa extracts on constitutive and  
30 induced cytokine production, parallel experiments were conducted using both



unstimulated (rested) PMBC's and cells which had been stimulated with the non-specific mitogen, phytohemagglutinin (PHA).

RT-PCR was used to measure variation in cytokine gene expression, whilst ELISA assays were used to assess cytokine protein secretion.

5        The cocoa procyanidin extract was purified from procyanidin-enriched cocoa powder according to the examples described in this application, and were analyzed by the method of Hammerstone *et al.*, "Identification of Procyanidins in Cocoa, *Theobroma cacao*, and Chocolate Using High-Performance Liquid Chromatography Mass Spectrometry", J. Agric. Food Chem., 47:2:490-496, 1999. Purified fractions of  
10 monomer through decamer were investigated. These purified fractions contained less than 0.5% (total w/w) of total alkaloids (theobromine and caffeine). The procyanidin composition, estimated by HPLC and molecular weights of these preparations, are shown in Table 3. All samples were suspended in RPMI 1640 with 10% heat inactivated fetal bovine serum. They were then diluted with the same medium to final  
15 concentrations of 25µg.

Peripheral blood from healthy volunteers was collected into sodium citrate-containing tubes and mixed 1:1 with Hanks' Balanced Salt Solution (HBSS; Gibco BRL) without calcium chloride, magnesium chloride, or magnesium sulfate. The diluted blood was then layered over an Accu-Paque (Accurate Chemical & Scientific  
20 Corp., Westbury, NY) gradient and centrifuged at 500 x g for 30 min. at room temperature. PBMC were harvested from the interface layer, washed twice with HBSS and then counted. The cells were resuspended in RPMI 1640 containing 10% fetal bovine serum and were supplemented with 0.1% of a 50 mg/ml gentamicin solution (Gibco BRL). PBMC concentration was adjusted to  $2.5 \times 10^6$  viable cells/ml  
25 after estimation of viability by Trypan Blue exclusion assay. Viability was consistently greater than 96%.

The cells were cultured for 8 hours at 37°C with 5% CO<sub>2</sub> in 48-well plates. In the first experiment  $5.0 \times 10^5$  cells (200 µl) were transferred into individual wells and a further 200 µl of media containing the different cocoa procyanidin fractions were  
30 added to each well. For control wells, media alone was added. In the second experiment this protocol was repeated but 20 µg/ml PHA were also added to each well. Control wells contained media plus PHA.

For the protein secretion assay, five hundred  $\mu\text{l}$  of a  $1.0 \times 10^6$  cell suspension were cultured with 500  $\mu\text{l}$  of the various cocoa treatments for 72 hours at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  in 48-well plates. Individual cocoa fractions at 25  $\mu\text{g}/\text{ml}$  were incubated in the presence and absence of PHA at 25  $\mu\text{g}/\text{ml}$ . All treatments were performed in  
5 duplicate.

(+)-Catechin and (-)-epicatechin (Sigma, St. Louis, MO) controls were also used in both assays at the same concentration as the individual cocoa fractions.

#### Expression Assay (RT-PCR)

Cells were harvested at 8 hours and transferred into 1.5 ml RNase-free  
10 Eppendorf tubes. Total cellular RNA was immediately extracted from cells using TRIzol Reagent (Gibco BRL, Gaithersburg, MD). Briefly, PBMC pellets were homogenized with 250  $\mu\text{l}$  TRIzol and chloroform (50  $\mu\text{l}$ ) was then added. After vigorously shaking the tubes for 15 seconds the cells were incubated for 3 minutes at room temperature and then centrifuged at  $12,000 \times g$  for 15 min at  $4^\circ\text{C}$ . The aqueous  
15 phase was transferred into another tube containing 125  $\mu\text{l}$  of isopropanol and 10  $\mu\text{g}$  of carrier tRNA (yeast tRNA, Gibco BRL). The samples were then incubated at room temperature for 10 minutes and again centrifuged at  $12,000 \times g$  for 10 min at  $4^\circ\text{C}$ . The supernatant was removed and the RNA pellet was washed with 250  $\mu\text{l}$  of 75% ethanol. The sample was mixed by vortexing and then centrifuged at  $7,500 \times g$  for 5  
20 min at  $4^\circ\text{C}$  before drying. The RNA pellet was dissolved in 12  $\mu\text{l}$  of DEPC-treated water. It was stored at  $-80^\circ\text{C}$  for up to 4 weeks without significant deterioration in message amplification (data not shown).

The RNA was then subjected to first-strand synthesis at  $42^\circ\text{C}$  for 50 minutes in a 20  $\mu\text{l}$  reaction-mixture containing 1  $\mu\text{g}$  RNA (5  $\mu\text{l}$ ), 25 mM tris-HCL (pH 8.3, at  
25 room temperature), 75 mM KCl, 3 mM  $\text{MgCl}_2$ , 10 mM DTT, 20 U RNAsin (Promega, Madison, WI), 1  $\mu\text{l}$  dNTP mix (10 mM of each of dATP, dCTP, dGTP, dTTP) (Pharmacia Biotech, Uppsala, Sweden), 0.5  $\mu\text{g}$  oligo-dTs, and 200 U Superscript II (Gibco BRL). After completion of first-strand synthesis, the cDNA was diluted 1:10 with DEPC water, and 2  $\mu\text{l}$  of the dilution was used for polymerase  
30 chain reaction (PCR). The final PCR mixture (25  $\mu\text{l}$ ) contained 0.2 mM of each dNTP, 25 ng of each specific primer, 1 U of Amplitaq Gold (Perkin Elmer, Foster

City, CA), and 2.5  $\mu$ l 10 X reaction buffer containing 15 mM  $MgCl_2$  (Perkin Elmer). Gene-specific primer sequences were chosen to cross introns in order to avoid amplifying genomic DNA. Primer sequences are shown below.

5

#### Cytokine Primers Used for Polymerase Chain Reaction

Cytokine		Fragment Size (b.p.)	Sequence
IL-2	5'	374	5'-ATGTACAGGATGCAACTCCTG
IL-2	3'		3'-CACATGAATGTTGTTTCAGATCCCT
GAPDH	5'	509	5'-ACCACCATGGAGAAGGCTGG
GAPDH	3'		3'-CTCAGTGTAGCCCAGGATGC
IL-1 $\beta$	5'	810	5'-ATGGCAGAAGTACCTGAGCTC
IL-1 $\beta$	3'		3'-TAGGAAGACACAAATTGCATGGTGAA
IL-6	for		5'-ATGAACTCCTTCTCCACAAGC
IL-6	rev		CTACATTTGCCGAAGAGCCCTCAGGCTGG ACTG
IL-4	for		5'-TTGAATATTTCTCTCTCATGATCGTCTT
IL-4	rev		5'-CTTGAATTCCTGTCCTGTGAAGG

PCR was performed in a Geneamp PCR System 9700 (Perkin Elmer) thermocycler with denaturation at 95°C for 45 sec, primer annealing for 2 min at 60°C and  
 10 extension at 72°C for 1 min. Cytokine gene-specific primers were multiplexed along with GAPDH 'housekeeping' gene primers, and the optimal number of cycles to identify the linear range of amplification was determined to be 32 cycles.

The PCR product was mixed thoroughly with 5  $\mu$ l of 10 X loading buffer (20% Ficoll 400, 0.1M  $Na_2EDTA$ , 1.0% SDS, 0.25% Bromophenol Blue, and 0.25%  
 15 Xylene cyanol). Ten  $\mu$ l of the mixture was then carefully loaded into a well of a 1.8% agarose LE (Boehringer Mannheim, Indianapolis, IN) gel prepared with TAE buffer containing 0.4  $\mu$ g/ml ethidium bromide. The gels were electrophoresed in TAE buffer at 80V for 60 min. The bands were visualized on a UV light box and photographed using a Polaroid film (Type 667). The positive image was computer-

scanned utilizing the Adobe Photoshop® 4.0 program and the intensity readings of each band were then calculated with the NIH Image 1.57 program.

Culture supernatant fractions were harvested after 72 h and were stored at -20°C until analysis by ELISA. Levels of cytokine in the supernatants of a  $1.0 \times 10^6$  cells/ml PBMC stimulated with cocoa fractions in the presence or absence of PHA were measured using the Quantikine Human Cytokine ELISA kits (R&D Systems, Minneapolis, MN). The lower limit of detection for the ELISA system was 3.9 pg/ml.

#### Data Analysis

Results are expressed as a percentage of the control values (i.e., without cocoa procyanidins) and, to eliminate variations caused by different yields of cDNA, results were normalized against the GAPDH housekeeping gene. The following equation was used for each sample:  $(\text{intensity reading of control GAPDH} / \text{intensity reading of treated GAPDH}) \times \text{intensity reading of treated IL-1}\beta = \text{adjusted intensity reading of treated IL-1}\beta$ . This adjusted value was then calculated as the percentage difference of the intensity reading of control IL-1 $\beta$ . In both experiments, i.e., with and without PHA, the effects of crude cocoa extracts, semi-purified cocoa extracts and isolated oligomeric fractions were investigated. Percentage changes were compared to 0 (i.e., control values-without cocoa extracts) using a one-tailed Students' T-test.

Significance was taken as  $0.05/n$ , where  $n$  is the number of comparisons made, using Bonferroni correction for small multiple comparisons. The means of triplicate data were taken for each volunteer and results are shown as mean  $\pm$  SEM of these volunteers.

#### Part I: IL-1 $\beta$

Transcription of IL-1 $\beta$  was assessed in unstimulated and PHA-stimulated PBMC from five subjects after an 8 hour treatment with 25  $\mu\text{g/ml}$  of the individual cocoa fractions. Their effects on constitutive IL-1 $\beta$  gene expression relative to media control are shown in Figure 1. The monomer, dimer, and trimer were able to augment IL-1 $\beta$  mRNA in 12 of 15 cultures, albeit the increase was slight in three of the subjects. Subject B appeared to have induced the highest stimulatory response with the monomeric fraction ( $+46\% \pm 10$ ). The tetramer showed only minimal changes,

ranging from -3% to +5%, in four of five subjects, with one subject (B) causing a 68% ( $\pm 5$ ) reduction in number of IL-1 $\beta$  transcripts. The hexamer and heptamer significantly augmented IL-1 $\beta$  expression in four of five subjects with an average increase of 23% and 20% respectively. Interestingly enough, the monomer showed  
5 slightly higher stimulatory response in four of the volunteers tested when compared to the effects displayed by (+)-catechin and (-)-epicatechin controls.

When PBMCs were co-incubated with PHA (at 25  $\mu$ g/ml) and individual cocoa fractions (at 25  $\mu$ g/ml), consistent effects were observed among the five subjects (Figure 7). The small oligomers (monomer through trimer) slightly  
10 suppressed PHA-induced expression of IL-1 $\beta$  in 13 of 15 cultures. Again, the tetramer showed minimal changes in four subjects (-4% to +4%) with one subject producing a 15%  $\pm 3$  reduction in IL-1 $\beta$  transcripts. The larger oligomers (hexamer through decamer) continually augmented IL-1 $\beta$  expression in 22 of 25 stimulated PBMCs cultures. The highest IL-1 $\beta$  stimulation caused by the heptamer, octamer,  
15 nonamer, and decamer were shown by subject A ( $52 \pm 10$ ,  $25 \pm 4$ ,  $23 \pm 7$ , and  $48 \pm 3$  respectively). The (+)-catechin and (-)-epicatechin controls showed similar effects as that seen with the monomer.

The secretion of IL-1 $\beta$  into culture supernatants were quantitated by ELISA following 72 hour incubation with the individual cocoa procyanidins. Again, cells  
20 were incubated with 25  $\mu$ g/ml of each fraction in the presence or absence of PHA. Their subsequent effects on IL-1 $\beta$  concentrations from the supernatants are shown in Figures 9 and 10 respectively. The smaller fractions alone (monomer through tetramer) showed significant reduction in 13 of 20 culture supernatants when compared to media control. In all five subjects, the tetramer appeared to be the  
25 fraction that produced the lowest values of IL-1 $\beta$  in culture supernatants. The tetramer caused a 67% to 75% reduction in the secretion of IL-1 $\beta$  in four of five subjects. In the presence of PHA, monomer through tetramer showed similar inhibition to a lesser extent when compared to PHA control. However, the other larger fractions (hexamer through decamer), markedly induced IL-1 $\beta$  secretion, either in the presence or  
30 absence of PHA. The two largest fractions, either the nonamer or decamer, stimulated the highest concentration of IL-1 $\beta$  with all subjects. The pentamer alone inhibited IL-

1 $\beta$  secretion in three subjects (13-47%), although the presence of PHA substantially augmented IL-1 $\beta$  concentration. Again, the monomer displayed a similar response as produced by the (+)-catechin and (-)-epicatechin.

Interleukin-1 $\beta$  is the prototypic pro-inflammatory cytokine, with broad  
5 physiological functions. In human PBMC, IL-1 $\beta$  synthesis is not only controlled at the transcriptional level, but translational modifications also appear to play an important role in regulating IL-1 $\beta$  production (Schindler *et al.*, J.Bio. Chem. 265 10232-1-237, 1990). Following translation, pro IL-1 $\beta$  remains primarily cytosolic until it is cleaved by the IL-1 $\beta$  converting enzyme and transported out of the cell as a  
10 mature IL-1 $\beta$  (Dinarello, Cytokine and Growth Factor Review 8 253-265, 1997).

Our analysis suggests that the incubation of PBMC with any one of the cocoa procyanidins can effectively modulate constitutive IL-1 $\beta$  gene expression. However, values of this stimulation were observed to vary between test subjects; this variation at the transcript level of IL-1 $\beta$  in healthy volunteers is expected. It is possible that the  
15 stability of the mRNA may have been a factor in the variability of IL-1 $\beta$  transcription. One study estimated the half-life of IL-1 $\beta$  mRNA to be 2 h in the absence, and 4.5 h in the presence, of LPS, a potent stimulator of IL-1 $\beta$  production. In addition, our investigation did not focus on kinetic responses of cocoa procyanidins, nor did we vary their dosage. Therefore fluctuating levels of IL-1 $\beta$  transcripts are conceivable.  
20 Moreover, adherence of PBMC to the plastic culture plates, which have been reported to induce IL-1 $\beta$  mRNA expression, could have masked any minor effects of the procyanidins.

The IL-1 $\beta$  protein levels following a 72 hour culture are particularly interesting. It was clear that size of the cocoa fraction dictated the response in  
25 secreted IL-1 $\beta$ . The smaller oligomeric cocoa procyanidins (monomer through tetramer) reduced IL-1 $\beta$  concentration in cultured supernatants of resting or PHA-stimulated cells (relative to respective controls). On the other hand, we observed significant increases by the larger oligomers (hexamer through decamer). Apparently, the pentamer seemed to be the only fraction affected by PHA. In the absence of  
30 PHA, the pentamer behaves like its smaller fraction, suppressing IL-1 $\beta$  secretion.

Identification of a polyphenolic fraction that modulates IL-1 $\beta$  production is important as this may have implications for other ingested plant products and could even allow the isolation of this fraction for pharmacological studies. We also studied the individual procyanidin fractions to delineate which of these were responsible for the observed inhibition. It is difficult to reconcile the efficacy of these oligomeric procyanidins, so we did not investigate synergy of the individual species. Judging from the data, it appears that small cocoa fractions (monomer through tetramer) are responsible for the down regulation IL-1 $\beta$  production in PBMC, while the larger oligomers (hexamer through decamer) increase synthesis.

These data support a role for dietary procyanidins from cocoa in the modulation of pro-inflammatory IL-1 $\beta$ .

#### Part II: Interleukin 2

T-cell activation is an important step in the initiation of an immunological response. Normally, resting T-cells do not contain constitutive levels of IL-2, Smith, K.A., "Interleukin-2: Inception, Impact And Implications", Science 240:1169, (1988). However, stimulation of T-cells by mitogens such as PHA results in the activation of a cascade of signaling events including the up-regulation of transcription factors, all of which lead to the transcription, translation and secretion of IL-2. (Han *et al.*, "TGF- $\beta$  Promotes IL-2 mRNA Expression Through The Up-Regulation Of NF- $\kappa$ B, AP-1 and NF-AT In EL4 Cells", J. Pharmacol. Exp. Therapeutics, 287:3:1105, 1998; Zhao *et al.*, "Propanil Affects Transcriptional And Post-Transcriptional Regulation Of IL-2 Expression In Activated EL-4 Cells", Toxicol. Appl. Pharmacol. 154:153, 1999). The subsequent interaction between IL-2 and its receptor on the T-cell promotes the cells to undergo cell cycle progression (Crabtree G.R., "Contingent Genetic Regulatory Events In T Lymphocyte Activation", Science 243:355, 1989). Hence the transcriptional regulation of IL-2 is critically involved in the control of T-cell expansion and the normal immune response (Smith, 1988, previously cited; Leonard *et al.*, "IL-2 Receptor Gene Expression In Normal Human T Lymphocytes", Proc. Natl. Acad. Sci. 82:6281, 1985).

It was found that incubating resting cells with the crude cocoa extract, the procyanidin-enriched cocoa extract, and the oligomeric fractions prepared from the cocoa extract had no effect on the basal levels of IL-2 mRNA.

However, various cocoa extracts were very active in stimulated cells.

- 5 Incubation with the procyanidin-enriched and the crude cocoa extracts both cause a pronounced reduction in IL-2 mRNA levels in PHA-stimulated cells (58%,  $p=0.0096$  and 67%,  $p=0.0002$  respectively). This is shown in Figure 11.

Interestingly, treatment with the individual oligomeric cocoa procyanidin fractions had markedly different effects on the PHA-stimulated cells. The monomeric  
10 fraction showed no significant modulation of IL-2 mRNA levels, whereas fractions containing the pentamer, hexamer and heptamer cause reductions of between 61-73% in IL-2 mRNA levels, as compared to an untreated cell culture. Allowing for multiple comparisons, modulation of IL-2 mRNA levels were significant for the pentamer ( $p=0.0022$ ), the hexamer ( $p=0.0111$ ) and the heptamer ( $p=0.0005$ ). Significance was  
15 taken as  $p<0.0125$ , ie,  $p<0.05/n$ , where  $n=4$ . Thus, it appears that procyanidin oligomers are responsible for the inhibition of PHA-stimulation of IL-2 transcription in PMBCs.

The finding that IL-2 expression by resting PBMCs is not effected by treatment with various cocoa fractions was to be expected since it had been reported  
20 by Farrar *et al.* in 1980 ("Macrophage-Independent Activation Of Helper T-Cells. Production Of IL-2", J. Immunol. 125:793) that IL-1, a macrophage-derived factor, is needed in the stimulation of IL-2 production from normal T-cells. Therefore, a lack of IL-2 expression in resting PBMCs was predicted from the findings of the study on modulation of constitutive IL-1 $\beta$  transcripts by cocoa fractions, which showed no  
25 effect.

These data support a role for dietary procyanidins from cocoa in the down-regulation of IL-2 at the mRNA level, allowing for the possibility of isolation or synthesis of the active oligomers for pharmacological studies. These data suggest that cocoa is an immune modulator, which may have therapeutic advantages in human  
30 diseases involving inappropriate activation of the immune system such as excema and arthritis. Further studies will investigate the control of secretion of this cytokine.



### Part III: Interleukin 4

In the resting cells, all the cocoa procyanidin oligomers caused a pronounced decrease in levels of IL-4 transcript, ranging from a 48% reduction for the dimer, to a 79% reduction for the heptamer. This is shown in Figure 12a. This trend of inhibition  
5 continued in the PHA-stimulated cells, as can be seen in Figure 12b. When the procyanidin oligomers were co-incubated with PHA, the larger oligomers (heptamer to decamer) suppressed virtually all IL-4 gene expression, whilst the smaller oligomers (monomer to tetramer) were able to maintain IL-4 gene expression above that of the medium control, but showed inhibition in comparison with the PHA  
10 control.

The protein secretion results appear to demonstrate more variation in effect between oligomers than the gene expression results. In the resting PBMCs, the monomer, trimer and tetramer showed decreased IL-4 secretion, with inhibition relative to the medium control ranging between 18% for the tetramer and 64% for the  
15 trimer. In contrast, the larger oligomers (pentamer through decamer) appeared to cause a marked increase in IL-4 protein secretion, with increases ranging between 3 and 9-fold over the medium control. These results are shown in Figure 13. However, when the cells were co-stimulated with PHA, the results were reversed with respect to the unstimulated cells: the monomer and dimer fractions enhanced IL-4 secretion by  
20 27% relative to the PHA control, whilst the trimer through decamer suppressed protein secretion by between 27% (trimer) and 70% (tetramer). These results are shown in Figure 14.

### Part IV: Interleukin 6

In the resting cells, it was shown that all the cocoa procyanidin oligomers, monomer through decamer, stimulated IL-6 gene expression to some extent, as can be  
25 seen in Figure 15a. However, when the cells were activated with PHA there was a general down-regulation of IL-6 expression with the larger oligomeric fraction (hexamer and above). This is shown in Figure 15b.

When IL-6 secretion was measured by ELISA there was a high constitutive  
30 amount of IL-6 production in the medium controls, with little or no increase seen with PHA stimulation. In both resting and stimulated cells, the only cocoa procyanidin

oligomeric fractions to have a significant effect on IL-6 secretion were the hexameric and nonameric fractions, both of which caused a significant decrease in protein secretion compared to the controls. These results are shown in Figures 16a and 16b.

5

**Table 3 COMPOSITION OF COCOA EXTRACTS USED.**

	Molecular Weight (Da)	Procyanidin Profile	%
Crude Cocoa Extract	N.D.	Monomer	44
		Dimer	16
		Trimer	13
		Tetramer	10
		Pentamer	7
		Hexamer-Decamer	10
Procyanidin-enriched Fraction	N.D.	Monomer	8
		Dimer	17
		Trimer	20
		Tetramer	21
		Pentamer	15
		Hexamer	11
		Heptamer-Decamer	9
Purified Fractions			
Monomer	280	Monomer	95
Pentamer	1442	Pentamer	93
Hexamer	1730	Hexamer	89
Heptamer/	2018	Heptamer	79
Hexamer		Hexamer	18

### Example 8

#### **Modulation of the Expression of the TNF- $\alpha$ Gene by Cocoa Procyanidins *in vitro***

Peripheral blood collected from healthy volunteers was used to isolate mononuclear cells (PBMCs) by standard Ficoll hypaque techniques. The concentration of the cell suspension was adjusted after as estimation of cell viability

by a Trypan blue exclusion assay. Approximately  $6 \times 10^5$  cells were treated in the following ways: media alone (baseline control), cocoa extract (prepared by the method out-lined in Example 6)- at five concentrations (.002, 0.02, 0.2, 2, 20  $\mu\text{g/ml}$ ), phytohemagglutinin (PHA; a mitogen that serves as a positive control), and PHA +  
5 cocoa extract. The cells were then incubated at 37 C , 5% CO<sub>2</sub>.

Following Incubation, cells were harvested at two time points: 1 and 8 hours. Total cellular RNA was immediately extracted from cells by using TRIzol Reagent (Gibco BRL, Gaithersburg, MD). The RNA was then subjected to first strand cDNA synthesis and RT-PCR analysis, exactly as described in Example 6.

10 For the proliferation assay cells were incubated 2-4 days and then pulsed with [3-H] Thymidine; cells were isolated and thymidine incorporated was then determined using a Beta-scan Scintillation Counter.

#### Results

The data collected to date shows that there is a strong effect of the crude cocoa  
15 extract on TNF- $\alpha$  production. The cocoa extract seems to be most effective at 0.2  $\mu\text{g/ml}$ , with production of TNF-  $\alpha$  ranging between about 3-4 times above baseline level at the 8 hour time point, as shown in Figure 17.

### **Example 9**

#### Tablet Formulations

20 A tablet formulation was prepared using high cocoa procyanidin cocoa solids obtained by methods described above, hereby incorporated herein by reference. Briefly, this edible material is prepared by a process which enhances the natural occurrence of the cocoa procyanidins in contrast to their levels found in traditionally processed cocoa, such that the ratio of the initial amount of the cocoa procyanidins  
25 found in the unprocessed bean to that obtained after processing is less than or equal to 2. For simplicity, this cocoa solids material is designated herein as CP-cocoa solids. The inventive compound or compounds, e.g., in isolated and/or purified form may be used in tablets as described in this Example, instead of or in combination with CP-cocoa solids.

A tablet formula comprises the following (percentages expressed as weight percent):

CP-cocoa solids	24.0%
4-Fold Natural vanilla extract (Bush Boake Allen)	1.5%
Magnesium stearate (dry lubricant)(AerChem, Inc.)	0.5%
Dipac tableting sugar (Amstar Sugar Corp.)	37.0%
Xylitol (American Xyrofin, Inc.)	<u>37.0%</u>
	100.0%

The CP-cocoa solids and vanilla extract are blended together in a food processor for 2 minutes. The sugars and magnesium stearate are gently mixed together, followed by blending in the CP-cocoa solids/vanilla mix. This material is run through a Manesty Tablet Press (B3B) at maximum pressure and compaction to produce round tablets (15mm x 5mm) weighing 1.5 - 1.8 gram. Another tablet of the above mentioned formula was prepared with a commercially available low fat natural cocoa powder (11% fat) instead of the CP-cocoa solids (11% fat). Both tablet formulas produced products having acceptable flavor characteristics and texture attributes.

An analysis of the two tablet formulas was performed using the procedures described in Example 6, Method 2. In this case, the analysis focused on the concentration of the pentamer and the total level of monomers and cocoa procyanidins where n is 2 to 12 which are reported below.

Tablet sample	pentamer ( $\mu\text{g/g}$ )	total ( $\mu\text{g/g}$ )	pentamer ( $\mu\text{g}/1.8\text{g}$ serving)	total ( $\mu\text{g}/1.8\text{g}$ serving)
tablet with CP-cocoa solids	239	8,277	430	14,989
tablet with commercial low fat cocoa powder	ND	868	ND	1563

ND = not detected

The data clearly showed a higher level of pentamer and total level of cocoa procyanidins in the CP-cocoa solids tablet than in the other tablet formula. Thus,

tablet formulas prepared with CP-cocoa solids are an ideal delivery vehicle for the oral administration of cocoa procyanidins, for pharmaceutical, supplement and food applications.

The skilled artisan in this area can readily prepare other tablet formulas covering a wide range of flavors, colors, excipients, vitamins, minerals, OTC medicaments, sugar fillers, UV protectants (e.g., titanium dioxide, colorants, etc.), binders, hydrogels, and the like except for polyvinyl pyrrolidone which would irreversibly bind the cocoa procyanidins or combination of compounds. The amount of sugar fillers may be adjusted to manipulate the dosages of the cocoa procyanidins or combination of compounds.

Many apparent variations of the above are self-evident and possible without departing from the spirit and scope of the example.

#### **Example 10**

##### **Capsule Formulations**

A variation of Example 15 for the oral delivery of the cocoa procyanidins is made with push-fit capsules made of gelatin, as well as soft sealed capsules made of gelatin and a plasticizer such as glycerol. The push-fit capsules contain the compound of the invention or combination of compounds or CP-cocoa solids as described in Example 16 in the form of a powder which can be optionally mixed with fillers such as lactose or sucrose to manipulate the dosages of the cocoa procyanidins. In soft capsules, the compound of the invention or combination of compounds or CP-cocoa solids are suspended in a suitable liquid such as fatty oils or cocoa butter or combinations therein. Since an inventive compound or compounds may be light-sensitive, e.g., sensitive to UV, a capsule can contain UV protectants such as titanium dioxide or suitable colors to protect against UV. The capsules can also contain fillers such as those mentioned in the previous Example.

Many apparent variations of the above are self-evident and possible to one skilled in the art without departing from the spirit and scope of the example.

#### **Example 11**

**Standard of Identity (SOI) and Non-Standard of Identity (non-SOI)**

##### **Dark and Milk Chocolate Formulations**

Formulations of the cocoa procyanidins or combination of compounds derived

by methods embodied in the invention can be prepared into SOI and non-SOI dark and milk chocolates as a delivery vehicle for human and veterinary applications. Reference is made to the International Appl. No. PCT/US97/15893 published as WO 98/09533 and based on the U.S. Application Serial No. 08/709,406, filed September 6, 1996, hereby incorporated herein by reference. The WO 98/09533 application relates to a method of producing cocoa butter and/or cocoa solids having conserved levels of the cocoa procyanidins from cocoa beans using a unique combination of processing steps. Briefly, the edible cocoa solids obtained by this process conserves the natural occurrence of the cocoa procyanidins in contrast to their levels found in traditionally processed cocoa, such that the ratio of the initial amount of the cocoa procyanidins found in the unprocessed bean to that obtained after processing is less than or equal to 2. For simplicity, this cocoa solids material is designated herein as CP-cocoa solids. The CP-cocoa solids are used as a powder or liquor to prepare SOI and non-SOI chocolates, beverages, snacks, baked goods, and as an ingredient for culinary applications.

The term "SOI chocolate" as used herein shall mean any chocolate used in food in the United States that is subject to a Standard of Identity established by the U.S. Food and Drug Administration under the Federal Food, Drug and Cosmetic Act. The U.S. definitions and standards for various types of chocolate are well established. The term "non-SOI chocolate" as used herein shall mean any nonstandardized chocolates which have compositions which fall outside the specified ranges of the standardized chocolates.

Examples of nonstandardized chocolates result when the cocoa butter or milk fat are replaced partially or completely; or when the nutritive carbohydrate sweetener is replaced partially or completely; or flavors imitating milk, butter, cocoa powder, or chocolate are added or other additions or deletions in the formula are made outside the U.S. FDA Standards of Identity for chocolate or combinations thereof.

As a confection, chocolate can take the form of solid pieces of chocolate, such as bars or novelty shapes, and can also be incorporated as a component of other, more complex confections where chocolate is optionally combined with any Flavor & Extract Manufacturers Association (FEMA) material, natural juices, spices, herbs and extracts categorized as natural-flavoring substances; nature-identical substances; and

artificial flavoring substances as defined by FEMA GRAS lists, FEMA and FDA lists, Council of Europe (CoE) lists, International Organization of the Flavor Industry (IOFI) adopted by the FAO/WHO Food Standard Programme, Codex Alimentarius, and Food Chemicals Codex and generally coats other foods such as caramel, nougat, fruit pieces, nuts, wafers or the like. These foods are characterized as microbiologically shelf-stable at 65-85°F under normal atmospheric conditions. Other complex confections result from surrounding with chocolate soft inclusions such as cordial cherries or peanut butter. Other complex confections result from coating ice cream or other frozen or refrigerated desserts with chocolate. Generally, chocolate used to coat or surround foods must be more fluid than chocolates used for plain chocolate solid bars or novelty shapes.

Additionally, chocolate can also be a low fat chocolate comprising a fat and nonfat solids, having nutritive carbohydrate sweetener(s), and an edible emulsifier. As to low fat chocolate, reference is made to U.S. Patent Nos. 4,810,516, 4,701,337, 5,464,649, 5,474,795, and WO 96/19923.

Dark chocolates derive their dark color from the amount of chocolate liquor, or alkalinized liquor or cocoa solids or alkalinized cocoa solids used in any given formulation.

Examples of formulations of SOI and non-SOI dark and milk chocolates are listed in Tables 18 and 19. In these formulations, the amount of the cocoa procyanidins present in CP-cocoa solids was compared to the cocoa procyanidins present in commercially available cocoa solids.

The following describes the processing steps used in preparing these chocolate formulations.

#### 25 Process for non-SOI Dark Chocolate

1. Keep all mixers and refiners covered throughout process to avoid light.
2. Batch all the ingredients excluding 40% of the free fat (cocoa butter and anhy. milk fat) maintaining temperature between 30-35 C.
3. Refine to 20 microns.
- 30 4. Dry conch for 1 hour at 35 C.
5. Add full lecithin and 10% cocoa butter at the beginning of the wet conche cycle; wet conche for 1 hour.

6. Add all remaining fat, standardize if necessary and mix for 1 hour at 35°C.
7. Temper, mould and package chocolate.

Process for SOI Dark Chocolate

1. Batch all ingredients excluding milk fat at a temperature of 60°C.
- 5 2. Refine to 20 microns.
3. Dry conche for 3.5 hours at 60°C.
4. Add lecithin and milk fat and wet conche for 1 hour at 60°C.
5. Standardize if necessary and mix for 1 hour at 35°C.

Temper, mould and package chocolate.

10 Process for non-SOI Milk Chocolate

1. Keep all mixers and refiners covered throughout process to avoid light.
2. Batch sugar, whole milk powder, malted milk powder, and 66% of the cocoa butter, conche for 2 hours at 75°C.
3. Cool batch to 35°C and add cocoa powder, ethyl vanillin, chocolate liquor and
- 15 21% of cocoa butter, mix 20 minutes at 35°C.
4. Refine to 20 microns.
5. Add remainder of cocoa butter, dry conche for 1.5 hour at 35°C.
6. Add anhy. milk fat and lecithin, wet conche for 1 hour at 35°C.
7. Standardize, temper, mould and package the chocolate.

20

Process for SOI Milk Chocolate

1. Batch all ingredients excluding 65% of cocoa butter and milk fat at a temperature of 60°C.
2. Refine to 20 microns.
- 25 3. Dry conche for 3.5 hours at 60°C.
4. Add lecithin, 10% of cocoa butter and anhy. milk fat; wet conche for 1 hour at 60°C.
5. Add remaining cocoa butter, standardize if necessary and mix for 1 hour at 35°C.
- 30 6. Temper, mould and package the chocolate.

The CP-cocoa solids and commercial chocolate liquors used in the



formulations were analyzed for the pentamer and total level of monomers and cocoa procyanidins where n is 2 to 12 as described in Method 2, Example 6 prior to incorporation in the formulations. These values were then used to calculate the expected levels in each chocolate formula as shown in Tables 18 and 19. In the cases  
5 for the non-SOI dark chocolate and non-SOI milk chocolate, their products were similarly analyzed for the pentamer, and the total level of monomers and the cocoa procyanidins where n is 2 to 12. The results appear in Tables 18 and 19.

The results from these formulation examples indicated that SOI and non-SOI dark and milk chocolates formulated with CP-cocoa solids contained approximately  
10 6.5 times more expected pentamer, and 3.5 times more expected total levels in the SOI and non-SOI dark chocolates; and approximately 4.5; 7.0 times more expected pentamer and 2.5; 3.5 times more expected total levels in the SOI and non-SOI milk chocolates, respectively.

Analyses of some of the chocolate products were not performed since the  
15 difference between the expected levels of the cocoa procyanidins present in finished chocolates prepared with CP-cocoa solids were dramatically higher than those formulas prepared with commercially available cocoa solids. However, the effects of processing was evaluated in the non-SOI dark and milk chocolate products. As shown in the tables, a 25-50% loss of the pentamer occurred, while slight differences in total  
20 levels were observed. Without wishing to be bound by any theory, it is believed that these losses are due to heat and/or low chain fatty acids from the milk ingredient (e.g. acetic acid, propionic acid and butyric acid) which can hydrolyze the oligomers (e.g. a trimer can hydrolyze to a monomer and dimer). Alternatively, time consuming processing steps can allow for oxidation or irreversible binding of the cocoa  
25 procyanidins to protein sources within the formula. Thus, the invention comprehends altering methods of chocolate formulation and processing to address these effects to prevent or minimize these losses.

The skilled artisan will recognize many variations in these examples to cover a wide range of formulas, ingredients, processing, and mixtures to rationally adjust the  
30 naturally occurring levels of the cocoa procyanidins for a variety of chocolate applications.

**Table 18. Dark Chocolate Formulas Prepared with non-Alkalized Cocoa****Ingredients**

Non-SOI Dark Chocolate Using CP-cocoa solids	SOI Dark Chocolate Using CP-Cocoa Solids	SOI Dark Chocolate Using Commercial Cocoa Solids
<b><u>Formulation:</u></b>	<b><u>Formulation:</u></b>	<b><u>Formulation:</u></b>
41.49 % Sugar 3% whole milk powder 26% CP-cocoa solids 4.5% com. liquor 21.75% cocoa butter 2.75% anhy. milk fat 0.01% vanillin 0.5% lecithin	41.49% sugar 3% whole milk powder 52.65% CP-liquor 2.35% anhy. milk fat 0.01% vanillin 0.5% lecithin	41.49% sugar 3% whole milk powder 52.65% com. liquor 2.35% anhy. milk fat 0.01% vanillin 0.5% lecithin
Total fat: 31%	Total fat: 31%	Total fat: 31%
Particle size: 20 microns	Particle size: 20 microns	Particle size: 20 microns

Expected Levels of pentamer and total oligomeric procyanidins (monomers and n = 2-

5 12; units of  $\mu\text{g/g}$ )

Pentamer: 1205	Pentamer: 1300	Pentamer: 185
Total: 13748	Total: 14646	Total: 3948

Actual Levels of pentamer and total oligomeric procyanidins (monomers and n = 2-

12; units of  $\mu\text{g/g}$ )

Pentamer: 561	Not performed	Not performed
Total: 14097		

**Table 18. Milk Chocolate Formulas Prepared with non-Alkalized Cocoa****Ingredients**

Non-SOI Milk Chocolate Using CP-cocoa solids	SOI Milk Chocolate Using CP-Cocoa Solids	SOI Milk Chocolate Using Commercial Cocoa Solids
<b><u>Formulation:</u></b>	<b><u>Formulation:</u></b>	<b><u>Formulation:</u></b>
46.9965 % Sugar 15.5% whole milk powder 4.5% CP-cocoa solids 5.5% com. liquor 21.4% cocoa butter 1.6% anhy. milk fat 0.035% vanillin 0.5% lecithin 4.0% malted milk powder	46.9965% sugar 15.5% whole milk powder 13.9% CP-liquor 1.6% anhy. milk fat 0.0035% vanillin 0.5% lecithin 17.5% cocoa butter 4.0% malted milk powder	46.9965% sugar 15.5% whole milk powder 13.9% com. liquor 1.60% anhy. milk fat 0.0035% vanillin 0.5% lecithin 17.5% cocoa butter 4.0% malted milk powder
Total fat: 31.75%	Total fat: 31.75%	Total fat: 31.75%
Particle size: 20 microns	Particle size: 20 microns	Particle size: 20 microns

Expected levels of pentamer and total oligomeric procyanidins (monomers and n = 2-

5 12; units of µg/g)

Pentamer: 225	Pentamer: 343	Pentamer: 49
Total: 2734	Total: 3867	Total: 1042

Actual levels of pentamer and total oligomeric procyanidins (monomers and n = 2-12;

units of µg/g)

Pentamer: 163	Not performed	Not performed
Total: 2399		

## Example 12

### Treatment of Asthma using Cocoa Procyanidins

Patients having a mild to moderate asthma are treated with cocoa procyanidin (300 mg/day) and the effect of the treatment is measured using peak flow meter and a life style questionnaire.

Patients, age 16-35, are recruited and interviewed to determine if they meet the following criteria: a) symptoms (cough, wheeze, chest tightness)  $\leq 2x/week$ ,  $\geq 4x/month$ ; b) current use of beta-2 agonist therapy only; c) no history of emergency room care or hospitalization; d) no use of prednisone in the past 3 months; and e) nonsmoker.

Patients meeting the following criteria are excluded from the study: 1. moderate or severe asthma by NAEPP 1997 revised guidelines; 2. pregnancy or lactation (there are unlikely to be any adverse effects of any of the trial arms but pregnancy and lactation may have an impact on asthma); 3. concurrent pulmonary disease: pulmonary hypertension, cystic fibrosis, sarcoidosis, bronchiectasis, hypersensitivity pneumonitis, restrictive lung disease, abnormal  $DLCO_{VA}$ ; 4. the following concurrent medical diagnoses: alcoholism, coronary artery disease, diabetes, HIV infection, chronic hepatitis, uncontrolled hypertension, chronic renal failure or a psychiatric disorder that is judged to make full participation difficult; 5. use of prednisone in the past 3 months, current use of inhaled corticosteroids, leukotriene antagonists, theophylline, or regular use of other systemic immunomodulating compounds; and 6. use of dietary supplements including herbal supplements, a high-dose multiple vitamins/minerals.

At initial study site visit (conducted one month prior to the beginning of the trial), the subjects sign an informed consent form, fill out a complete medical history/review form and undergo a complete physical exam except for the breast/genital/pelvic/rectal exam. The results of the physical is consistent with mild asthma according to the 1997 NIH NAEPP guidelines (baseline values are  $FEV_1 > 70\%$  predicted,  $FEV_1/FVC$  ratio  $> 75\%$ ).

Recruitment efforts are directed to all income and ethnic groups. Pre-trial computerized randomization of qualified subjects are utilized. A central coordinator

without patient contact is contacted for assignment within each clinical trial arm to placebo or active therapy (that are double-blinded to the investigators and assistants conducting follow-up visits).

There are 24 patients enrolled in the study. Subjects are randomly assigned to  
5 receive either the high cocoa polyphenol Dove chocolate bar (available from Mars, Inc., to achieve 300 mg cocoa polyphenol/day) or a polyphenolic depleted Dove chocolate bar control (available from Mars, Inc.). The bars are consumed for 3 months, followed by a 4 week wash-out and a cross-over arm. To monitor compliance, Dove bar wrappers are returned monthly for a count. Subjects are  
10 requested to be honest in returning bars not taken. Asthma is evaluated by clinical markers (asthma symptom diary, and morning and evening peak-flow daily and asthma quality of life (QOL) questionnaire weekly).

What is Claimed:

1. A method for treating asthma by administering, to a mammal suffering from asthma, a cocoa procyanidin in an amount effective to treat the asthma.
2. The method of claim 1, wherein the mammal is a human.
- 5 3. The method of claim 1 or 2, wherein the cocoa procyanidin is in a procyanidin containing cocoa extract.
4. The method of claim 1 or 2, wherein the cocoa procyanidin is in a cocoa extract prepared by the step of removing theobromine and/or caffeine from a crude procyanidin containing cocoa extract.
- 10 5. The method of claim 1 or 2, wherein the cocoa procyanidin comprises procyanidin monomers and oligomers.
6. The method of claim 1 or 2, wherein the cocoa procyanidin comprises at least 50% by weight of oligomers.
7. The method of claim 1 or 2, wherein the cocoa procyanidin comprises at least one  
15 of oligomers 3-10.
8. The method of claim 1 or 2, wherein the cocoa procyanidin comprises a dimer.
9. The method of claim 1 or 2, wherein the cocoa procyanidin is in an ingredient processed to preserve the procyanidin levels.
10. A method for preventing or reducing the risk of a viral infection by administering,  
20 to a mammal at risk of a viral infection, a cocoa procyanidin in an amount effective to prevent or reduce the risk of a viral infection.
11. The method of claim 10, wherein the mammal is a human.
12. The method of claim 11, wherein the virus is a rhinovirus.
13. The method of claim 11, wherein the virus is a myxovirus.
- 25 14. The method of claim 10 or 11, wherein the cocoa procyanidin is in a procyanidin containing cocoa extract.
15. The method of claim 10 or 11, wherein the cocoa procyanidin is in a cocoa extract prepared by the step of removing theobromine and/or caffeine from a crude procyanidin containing cocoa extract.
- 30 16. The method of claim 10 or 11, wherein the cocoa procyanidin comprises procyanidin monomers and oligomers.

17. The method of claim 10 or 11, wherein the cocoa procyanidin comprises at least 50% by weight of oligomers.
18. The method of claim 10 or 11, wherein the cocoa procyanidin comprises at least one of oligomers 3-10.
- 5 19. The method of claim 10 or 11, wherein the cocoa procyanidin comprises a dimer.
20. The method of claim 10 or 11, wherein the cocoa procyanidin is in an ingredient processed to preserve the procyanidin levels.
21. A method for enhancing an immune response to, or treating, a viral infection by administering, to a mammal infected with a virus, a cocoa procyanidin in an  
10 amount effective to enhance the immune response or to treat the viral infection,
22. The method of claim 21, wherein the mammal is a human.
23. The method of claim 22, wherein the virus is a rhinovirus.
24. The method of claim 22, wherein the virus is a myxovirus.
25. The method of claim 21 or 22, wherein the cocoa procyanidin is in a procyanidin  
15 containing cocoa extract.
26. The method of claim 21 or 22, wherein the cocoa procyanidin is in a cocoa extract prepared by the step of removing theobromine and/or caffeine from a crude procyanidin containing cocoa extract.
27. The method of claim 21 or 22, wherein the cocoa procyanidin comprises  
20 procyanidin monomers and oligomers.
28. The method of claim 21 or 22, wherein the cocoa procyanidin comprises at least 50% by weight of oligomers.
29. The method of claim 21 or 22, wherein the cocoa procyanidin comprises at least one of oligomers 3-10.
- 25 30. The method of claim 21 or 22, wherein the cocoa procyanidin comprises a dimer.
31. The method of claim 21 or 22 wherein the cocoa procyanidin is in an ingredient processed to preserve the procyanidin levels.
32. A nasal delivery dosage form comprising cocoa procyanidin monomers and/or oligomers.
- 30 33. A product comprising a cocoa procyanidin containing composition and a label instructing use for at least one of the following: treating asthma, preventing or

reducing the risk of a viral infection, enhancing an immune response to a viral infection or treating a viral infection.

34. The use of a cocoa procyanidin for the manufacture of a medicament, food or dietary supplement for treating asthma.
- 5 35. The use according to claim 34, wherein the cocoa procyanidin is in a form of a procyanidin containing cocoa extract.
36. The use according to claim 34, wherein the cocoa procyanidin is in a form of a cocoa extract prepared by the step of removing theobromine and/or caffeine from a crude procyanidin containing cocoa extract.
- 10 37. The use according to claim 34, wherein the cocoa procyanidin comprises procyanidin monomers and oligomers.
38. The use according to claim 34, wherein the cocoa procyanidin comprises at least 50% by weight of oligomers.
39. The use according to claim 34, wherein the cocoa procyanidin comprises at least one of oligomers 3-10.
- 15 40. The use according to claim 34, wherein the cocoa procyanidin comprises a dimer.
41. The use according to claim 34, wherein the cocoa procyanidin is in an ingredient processed to preserve the procyanidin levels.
42. The use of a cocoa procyanidin for the manufacture of a medicament, food or dietary supplement for preventing or reducing the risk of a viral infection.
- 20 43. The use according to claim 42, wherein the cocoa procyanidin is in a form of a procyanidin containing cocoa extract.
44. The use according to claim 42, wherein the cocoa procyanidin is in a form of a cocoa extract prepared by the step of removing theobromine and/or caffeine from a crude procyanidin containing cocoa extract.
- 25 45. The use according to claim 42, wherein the cocoa procyanidin comprises procyanidin monomers and oligomers.
46. The use according to claim 42, wherein the cocoa procyanidin comprises at least 50% by weight of oligomers.
- 30 47. The use according to claim 42, wherein the cocoa procyanidin comprises at least one of oligomers 3-10.
48. The use according to claim 42, wherein the cocoa procyanidin comprises a dimer.



49. The use according to claim 42, wherein the cocoa procyanidin is in an ingredient processed to preserve the procyanidin levels.
50. The use of a cocoa procyanidin for the manufacture of a medicament, food or dietary supplement for enhancing an immune response to, or treating, a viral  
5 infection.
51. The use according to claim 50, wherein the cocoa procyanidin is in a form of a procyanidin containing cocoa extract.
52. The use according to claim 50, wherein the cocoa procyanidin is in a form of a cocoa extract prepared by the step of removing theobromine and/or caffeine from  
10 a crude procyanidin containing cocoa extract.
53. The use according to claim 50, wherein the cocoa procyanidin comprises procyanidin monomers and oligomers.
54. The use according to claim 50, wherein the cocoa procyanidin comprises at least 50% by weight of oligomers.
- 15 55. The use according to claim 50, wherein the cocoa procyanidin comprises at least one of oligomers 3-10.
56. The use according to claim 50, wherein the cocoa procyanidin comprises a dimer.
57. The use according to claim 50, wherein the cocoa procyanidin is in an ingredient processed to preserve the procyanidin levels.
- 20 58. The use of procyanidin monomers and/or oligomers isolated from tomato, peanut, almond, apple, cranberry or blueberry for the manufacture of a medicament, food or dietary supplement for treating asthma.
59. The use of procyanidin monomers and/or oligomers isolated from tomato, peanut, almond, apple, cranberry or blueberry for the manufacture of a medicament, food  
25 or dietary supplement for preventing or reducing the risk of a viral infection.
60. The use of procyanidin monomers and/or oligomers isolated from tomato, peanut, almond, apple, cranberry or blueberry for the manufacture of a medicament, food or dietary supplement for enhancing an immune response to, or treating, a viral infection.
- 30 61. The use of a methylated procyanidin for the manufacture of a medicament, food or dietary supplement for treating asthma.

62. The use of a methylated procyanidin for the manufacture of a medicament, food or dietary supplement for preventing or reducing the risk of a viral infection.
63. The use of a methylated procyanidin for the manufacture of a medicament, food or dietary supplement for enhancing an immune response to, or treating, a viral  
5 infection.

Fig. 1. Summary of the current purification protocol

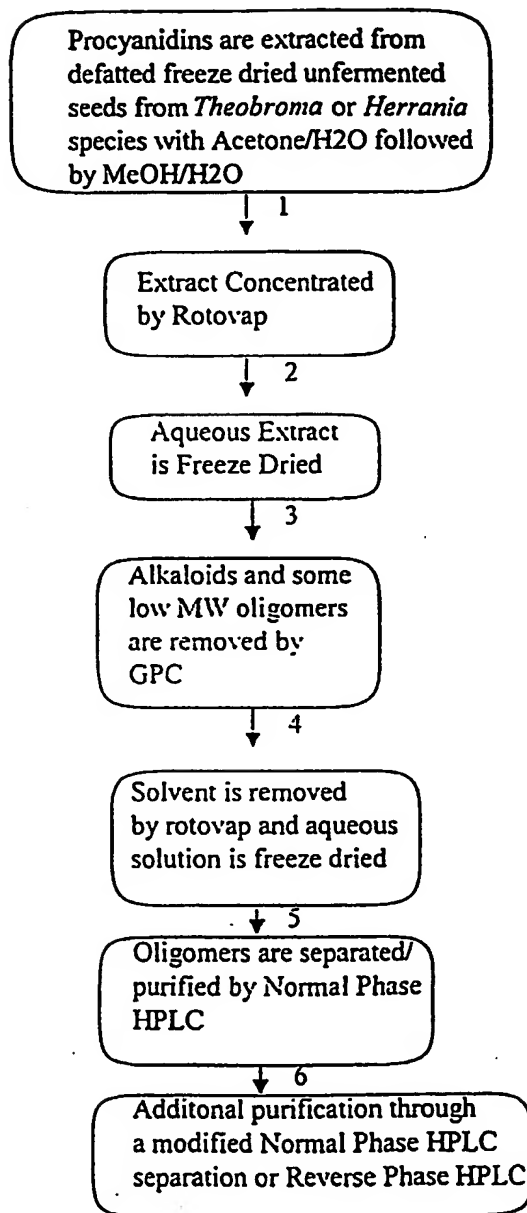


Figure 2

## Experimental Design

### Isolate PBMC

(Adjust concentration after estimation of viability by trypan blue exclusion assay.)

#### Treatments

- 1) media alone (baseline control)
- 2) cocoa extract
- 3) PHA ( + control)
- 4) PHA + cocoa extract

### ELISA Assay for Protein

incubate @ 37 C,  
5% CO<sub>2</sub>, 72 hrs

ELISA assay for  
cytokine secretion

### RT-PCR

harvest cells @  
8 hr time point

RNA Isolation

RNA

Reverse  
Transcriptase

cDNA

Real Time PCR with cytokine primers  
IL-1, IL-2, IL-4, and IL-6

Data analysis

Figure 3

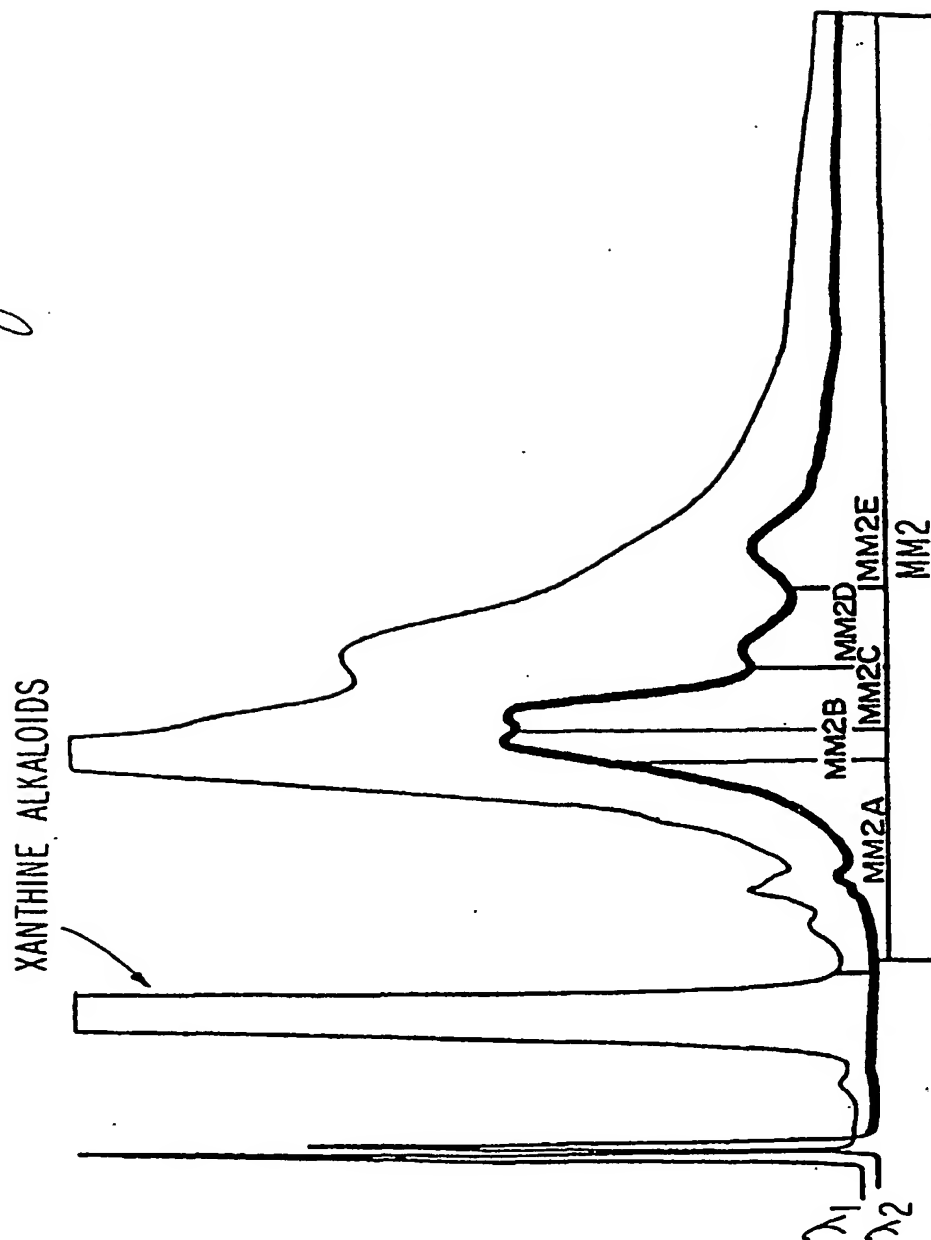


FIG. 4

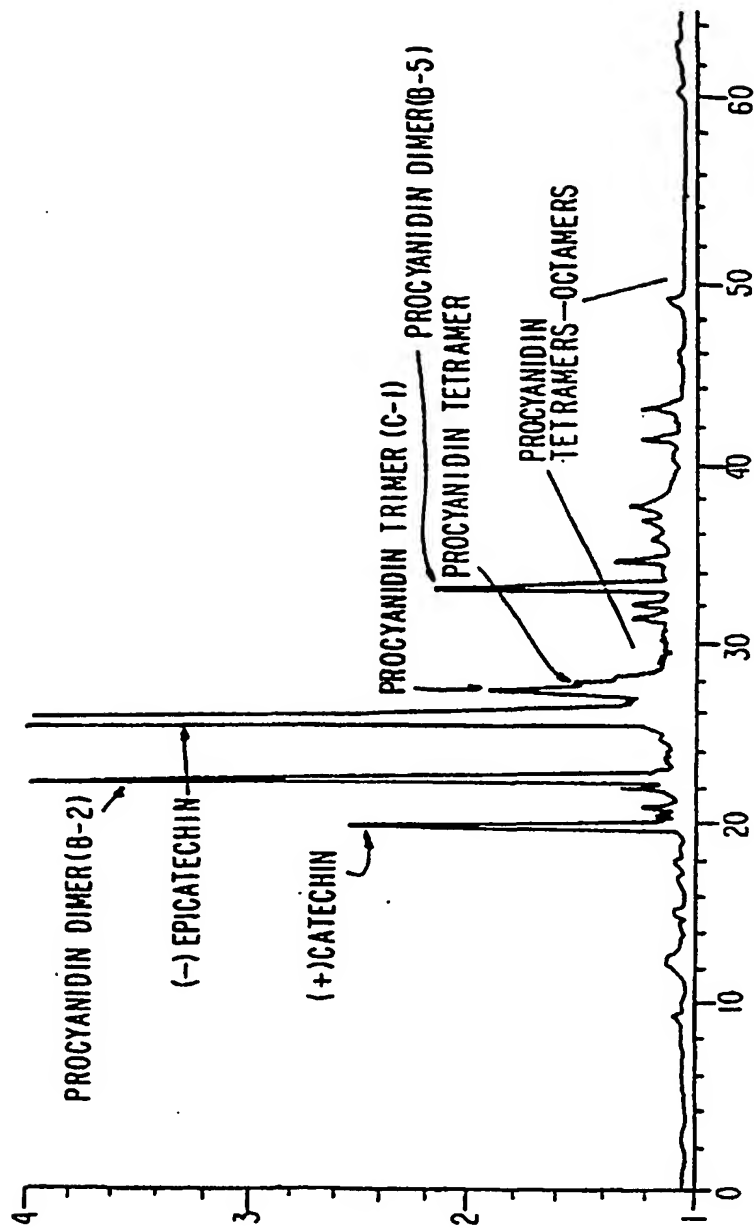


FIG. 5

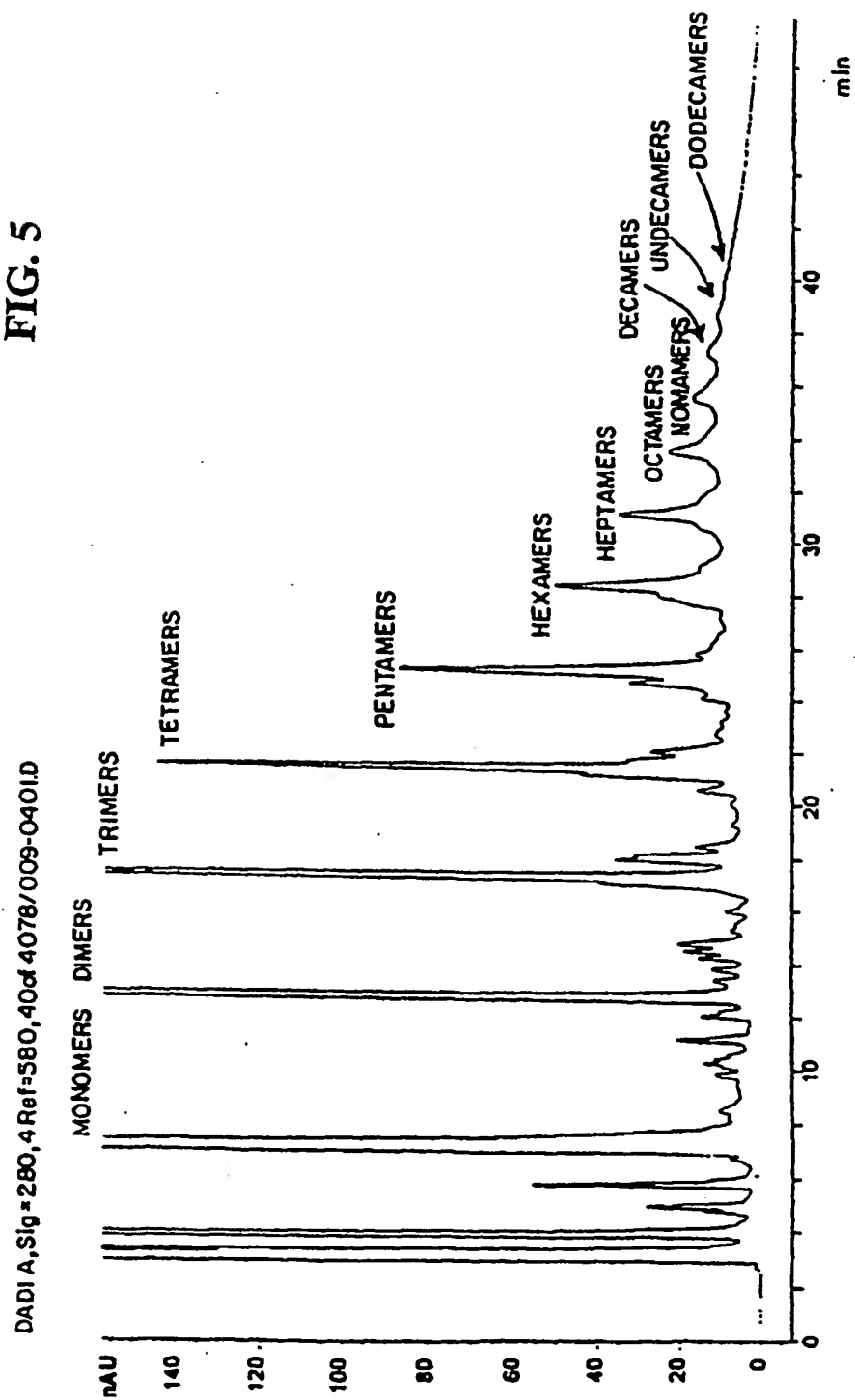
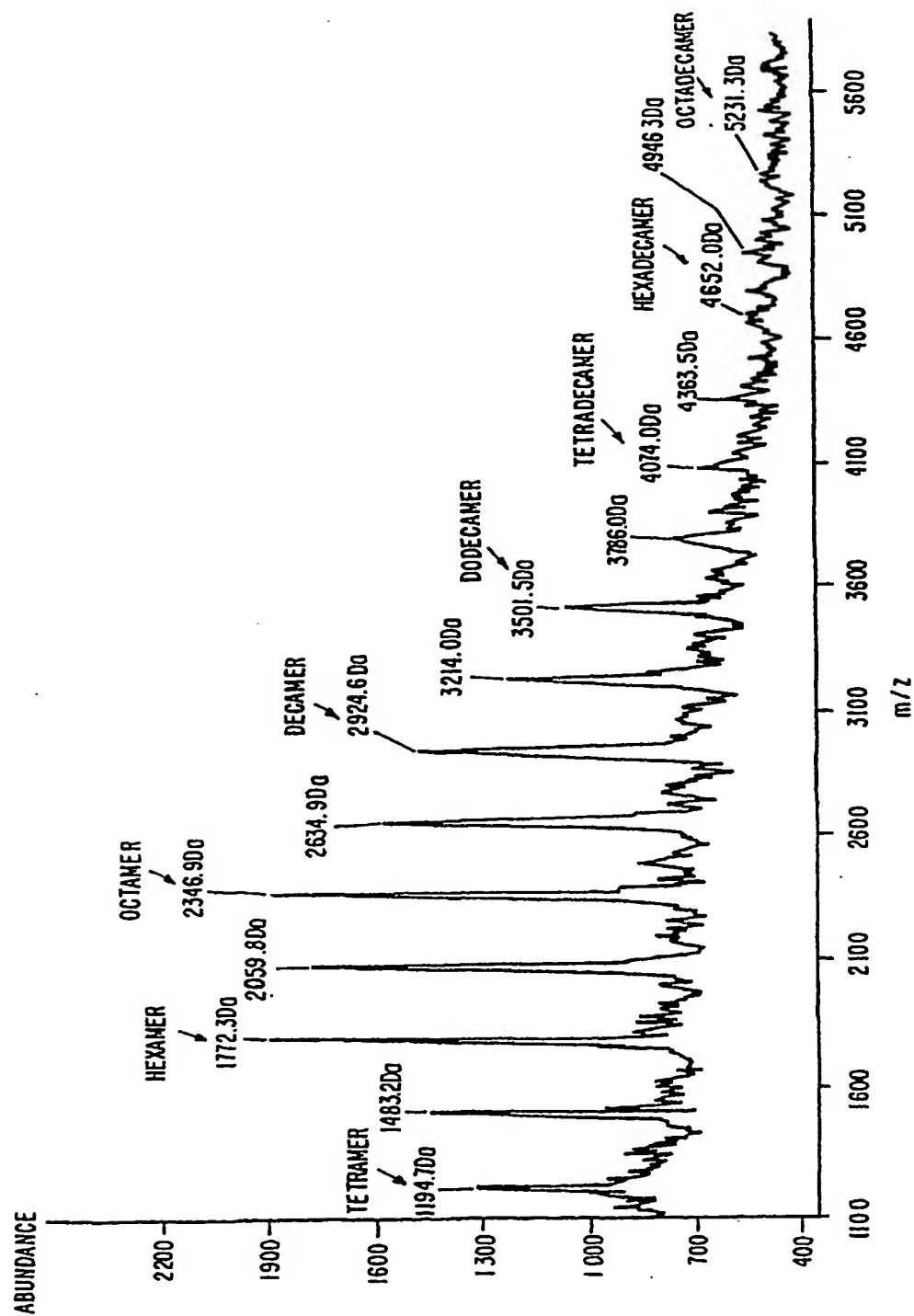


FIG. 6





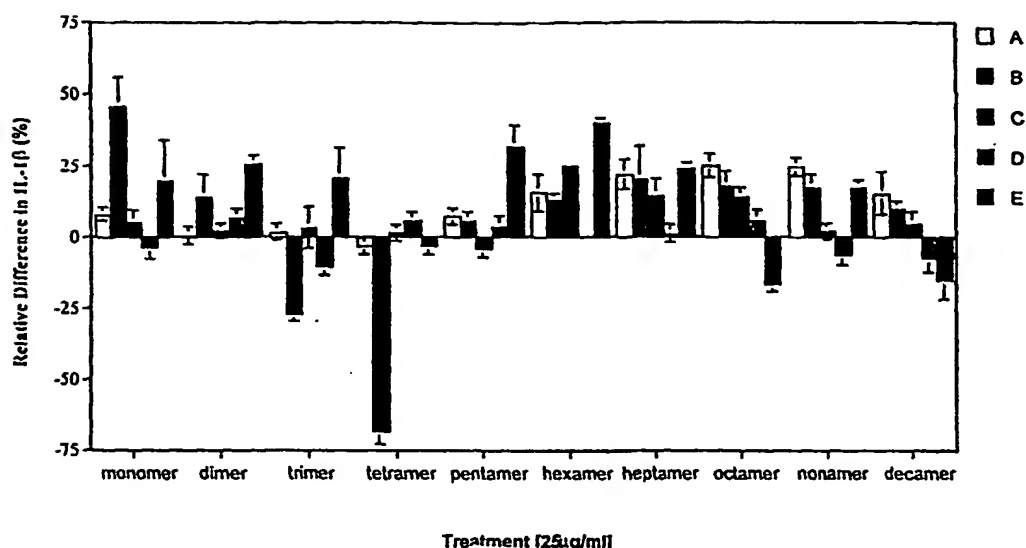


Figure 7

Effects of cocoa procyanidin oligomeric fractions on the transcription of IL-1 $\beta$  from human PBMC of five subjects (A-E). The cells were incubated with 25  $\mu$ g/ml of individual fractions for 8 hours at 37°C with each fraction. Treatments are compared to media alone, with each value representing the average of triplicate cultures (mean $\pm$ SEM). The monomer showed slightly higher stimulatory response in four of the volunteers tested when compared to the effects displayed by (+)-catechin and (-)-epicatechin controls (data not shown).

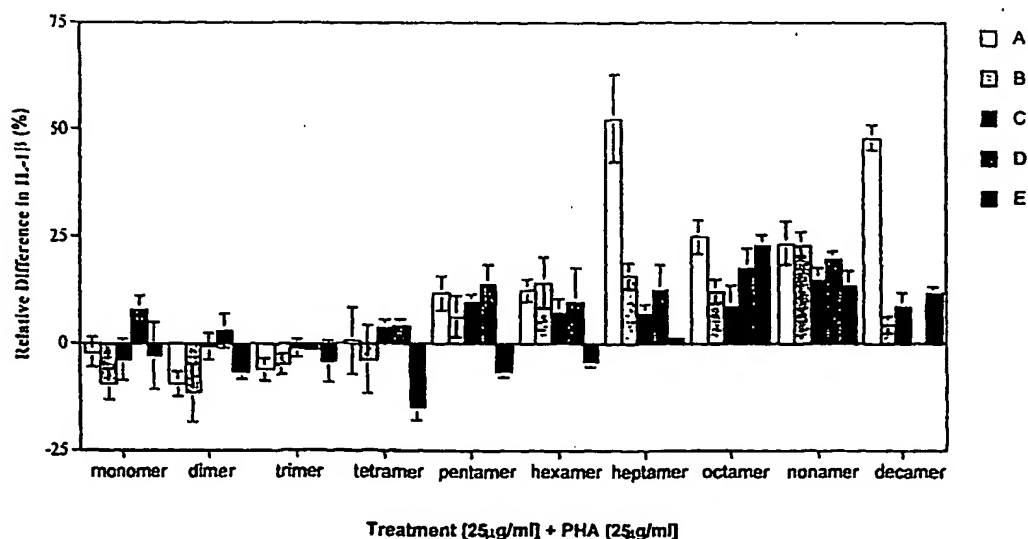


Figure 8

Effects of cocoa procyanidin oligomeric fractions on the transcription of IL-1 $\beta$  from PHA-stimulated PBMC of five subjects (A-E). The cells were co-incubated with 25  $\mu$ g/ml of individual fractions and 25  $\mu$ g/ml of PHA for 8 hours at 37°C. Treatments are compared to PHA control, with each value representing the average of triplicate cultures (mean $\pm$ SEM). (+)-Catechin and (-)-epicatechin controls were also tested at 25  $\mu$ g/ml and showed similar effects as produced by the monomer (data not shown).

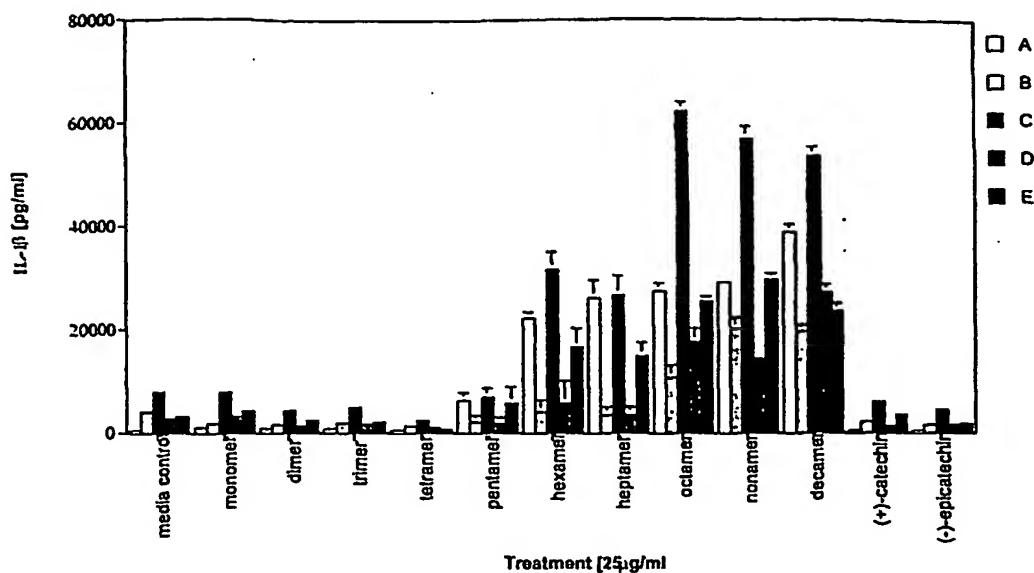


Figure 9

Effects of cocoa procyanidin oligomeric fractions on the protein secretion of IL-1 $\beta$  from human PBMC of six subjects (A-E). The cells were incubated with 25  $\mu$ g/ml of individual fractions for 72 hours at 37°C before the supernatants were removed for ELISA analysis. The values are expressed in concentration (pg/ml) and represent the average of duplicate cultures (mean $\pm$ SEM). (+)-Catechin and (-)-epicatechin controls were also tested at 25  $\mu$ g/ml.

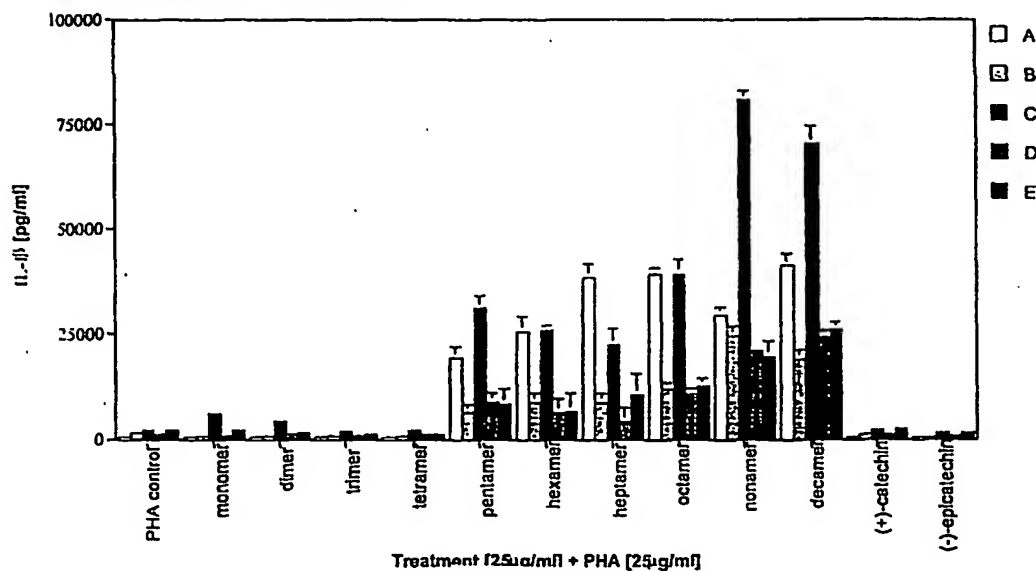
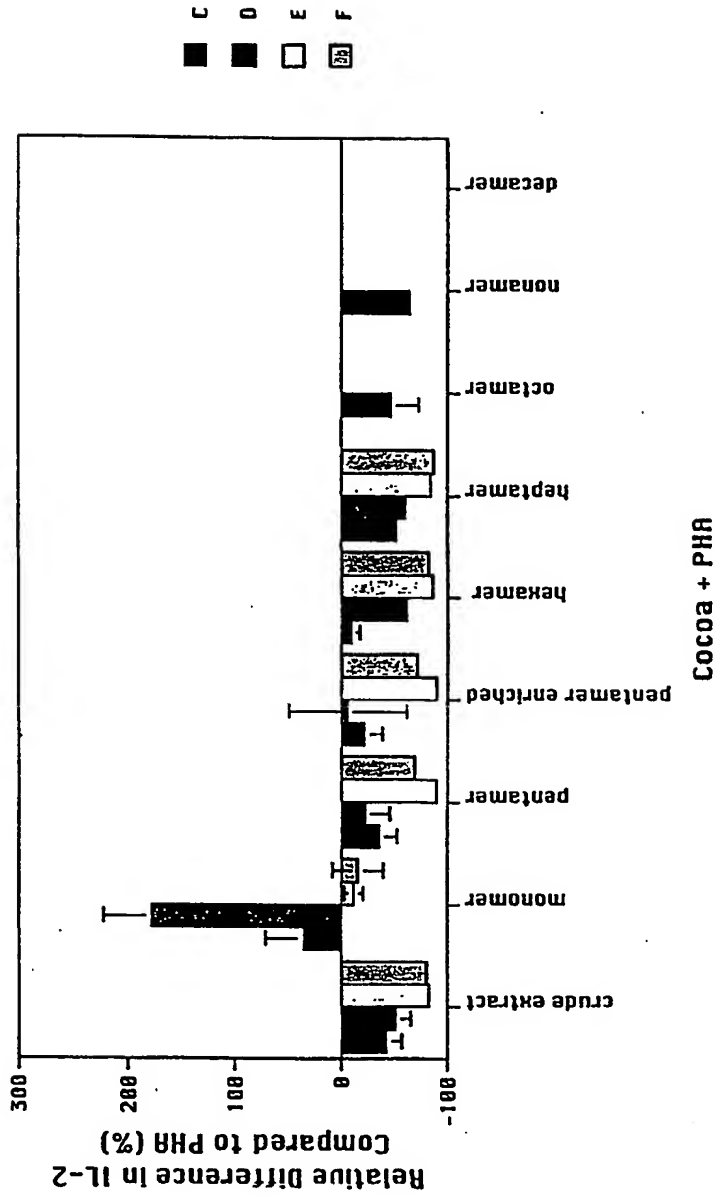


Figure 10

Effects of cocoa procyanidin oligomeric fractions on the protein secretion of IL-1 $\beta$  from PHA-stimulated PBMC of five subjects (A-E). The cells were co-incubated with 25  $\mu$ g/ml of individual fractions and 25  $\mu$ g/ml of PHA for 72 hours at 37°C before the supernatants were removed for ELISA analysis. The values are expressed in concentration (pg/ml) and represent the average of duplicate cultures (mean $\pm$ SEM). (+)-Catechin and (-)-epicatechin controls were also tested at 25  $\mu$ g/ml.

Figure 11

The Effect of Cocoa Oligomers on IL-2  
Expression in PHA Stimulated PBMC

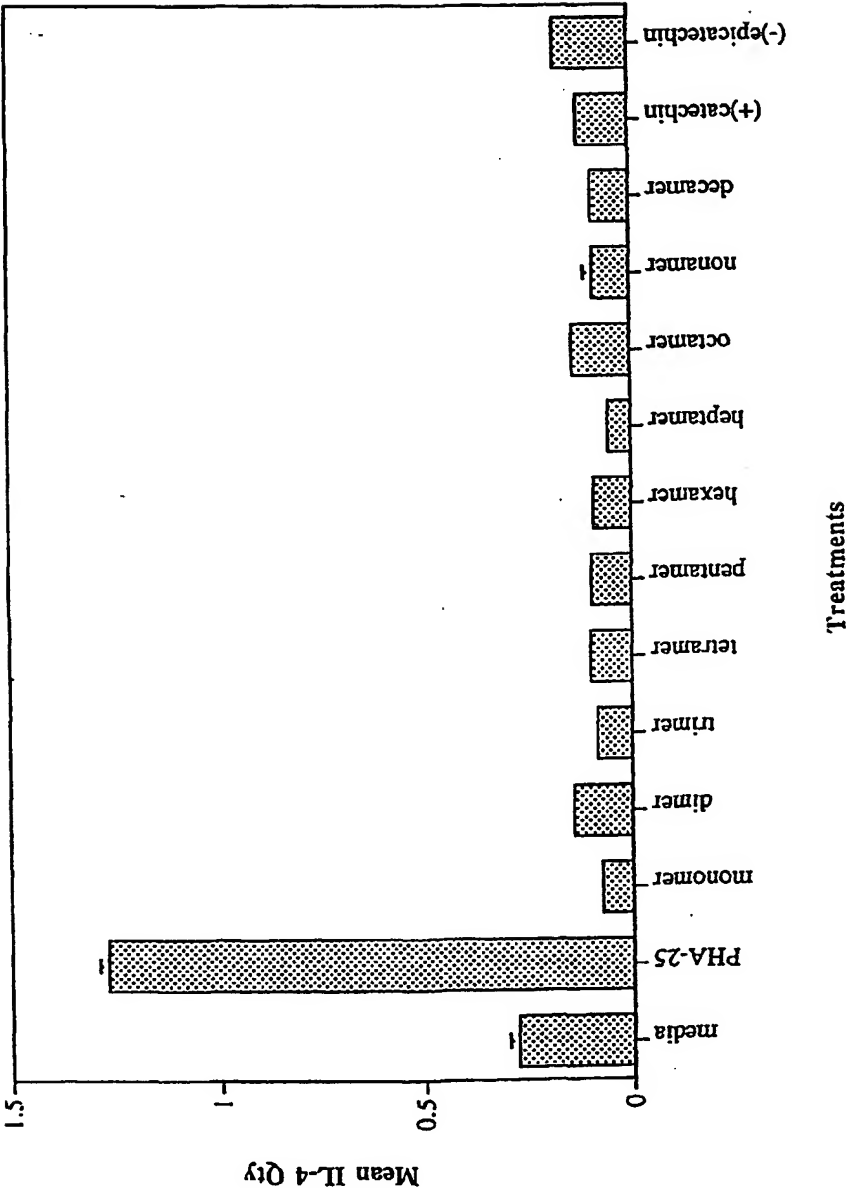


The crude and pentamer enriched extracts were able to demonstrate a pronounced reduction of IL-2 transcription in PHA-stimulated cells by 67% and 58% respectively.

The monomeric fraction did not alter IL-2 expression, while the pentamer, hexamer, and heptamer caused a 61-73% inhibition in PHA-stimulated cells.

Figure 12A

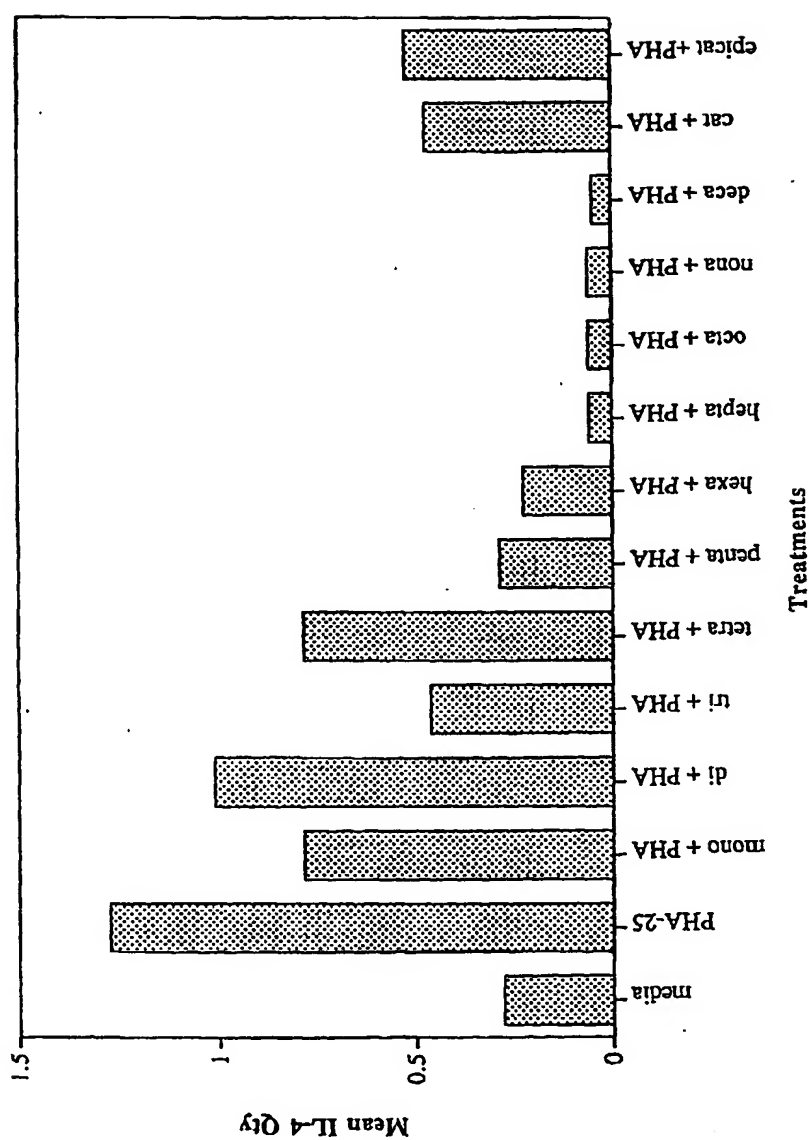
Effect of Cocoa Oligomers [25ug/ml] on IL-4 mRNA  
(following 8 hr incubation)



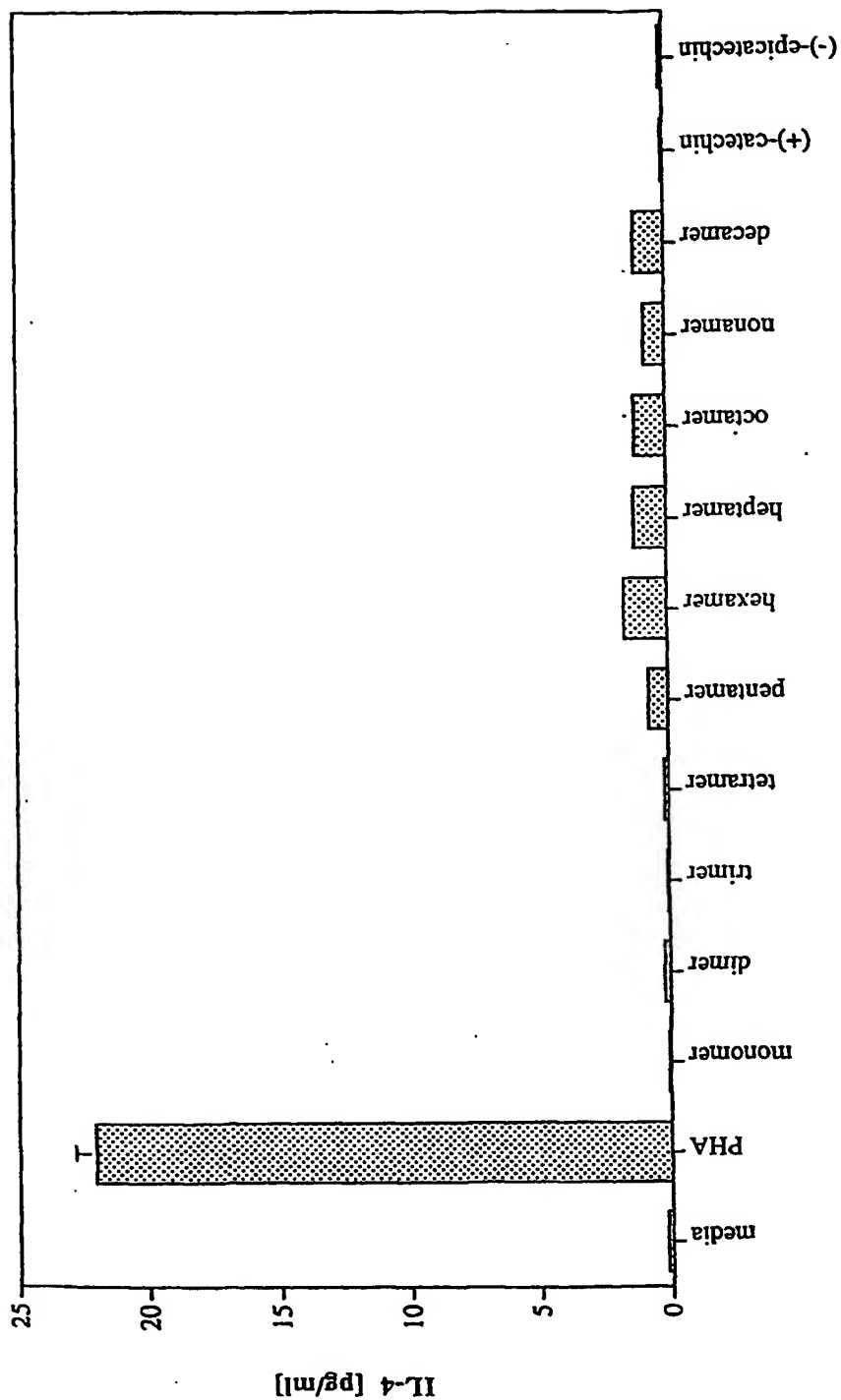
All cocoa oligomers showed a pronounced decrease in IL-4 transcripts ranging from 48% (dimer) to 79% (heptamer) relative to the media control.

Figure 12B

Effect of Cocoa Oligomers [25ug/ml] Co-incubated with PHA[25ug/ml] on IL4 mRNA  
(following 8 hr incubation)



When cocoa fractions were co-incubated with PHA, the larger oligomers (heptamer-decamer) suppressed nearly all IL-4 expression. The smaller oligomers (mono-tetramer) were still able to maintain IL-4 expression above media but inhibition relative to PHA stimulation (20-64%).

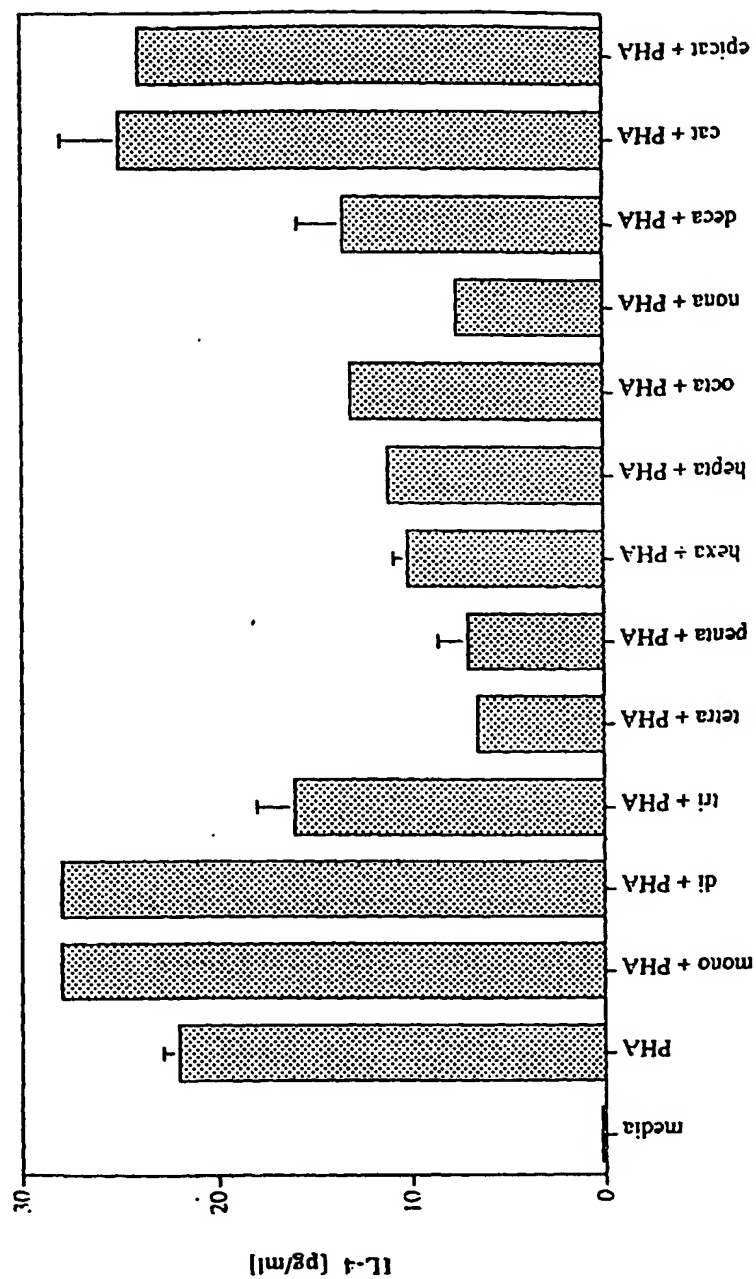


#### Treatments

The monomer, trimer, and tetramer showed decreased IL-4 secretion relative to the media control (18%;tetramer-64%;trimer).  
The larger oligomers (penta-decamer) markedly increased IL-4 protein production (3-9 fold over media).

**FIG. 13**

# Effect of Cocoa Oligomers [25ug/ml] Co-incubated with PHA [25ug/ml] on IL-4 Secretion (72hr)

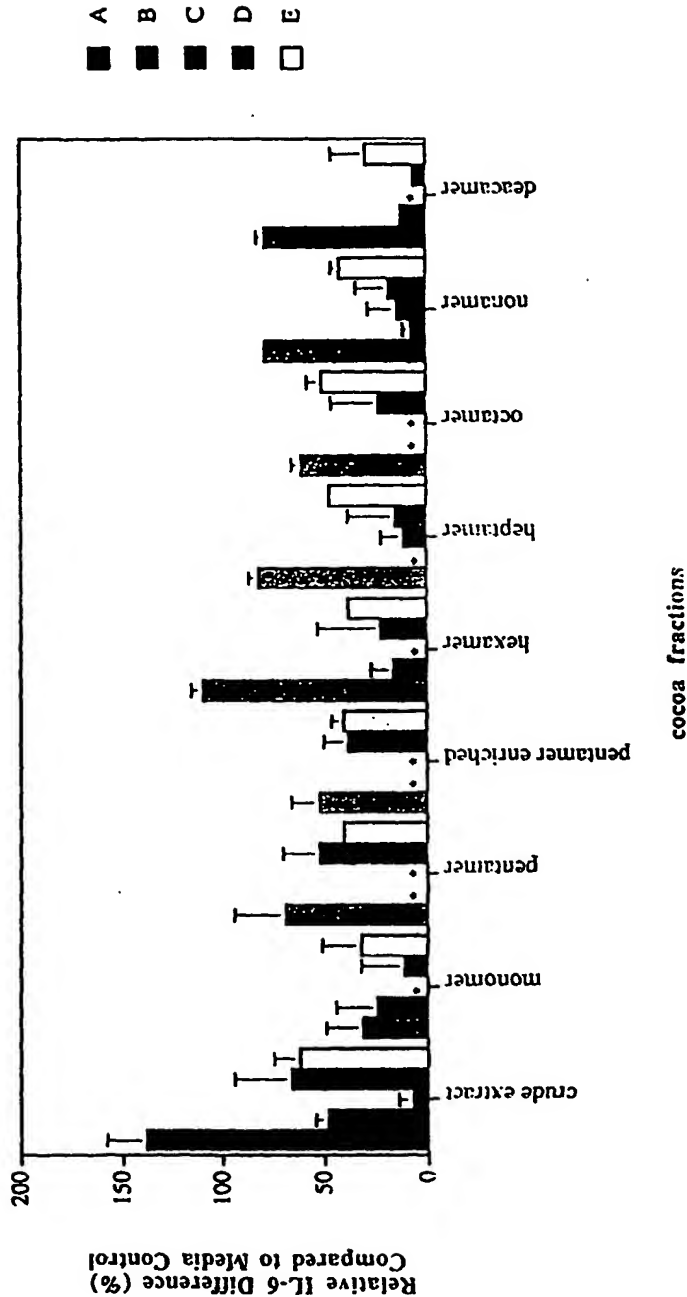


Treatments

When the cells were co-stimulated with PHA, the monomer and dimer fractions both enhanced IL-4 secretion by 27% relative to the PHA only control.  
The rest of the oligomers suppressed protein secretion by 27%; trimer-70%; tetramer).

FIG. 14

Effect of Cocoa Oligomers on IL-6 Expression  
(following 8hr incubation)

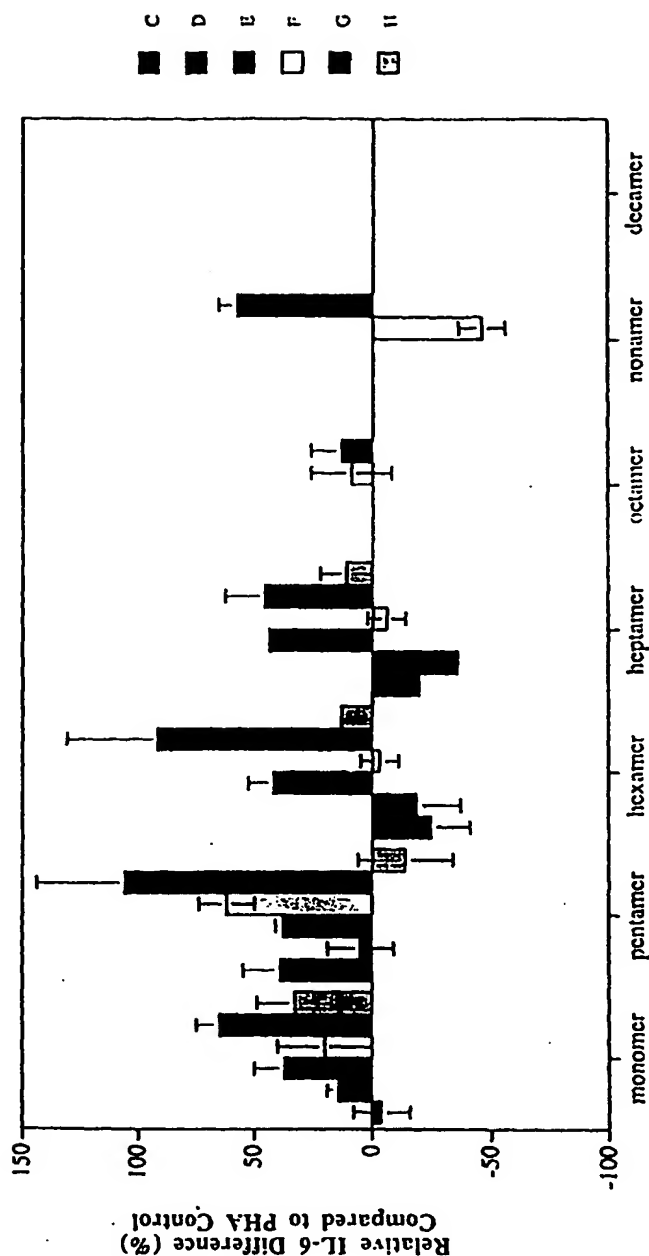


All cocoa extracts stimulated IL-6 mRNA expression to some degree in most individuals.

FIG. 15A



# Effect of Cocoa Oligomers on IL-6 Expression in PHA-stimulated Cells



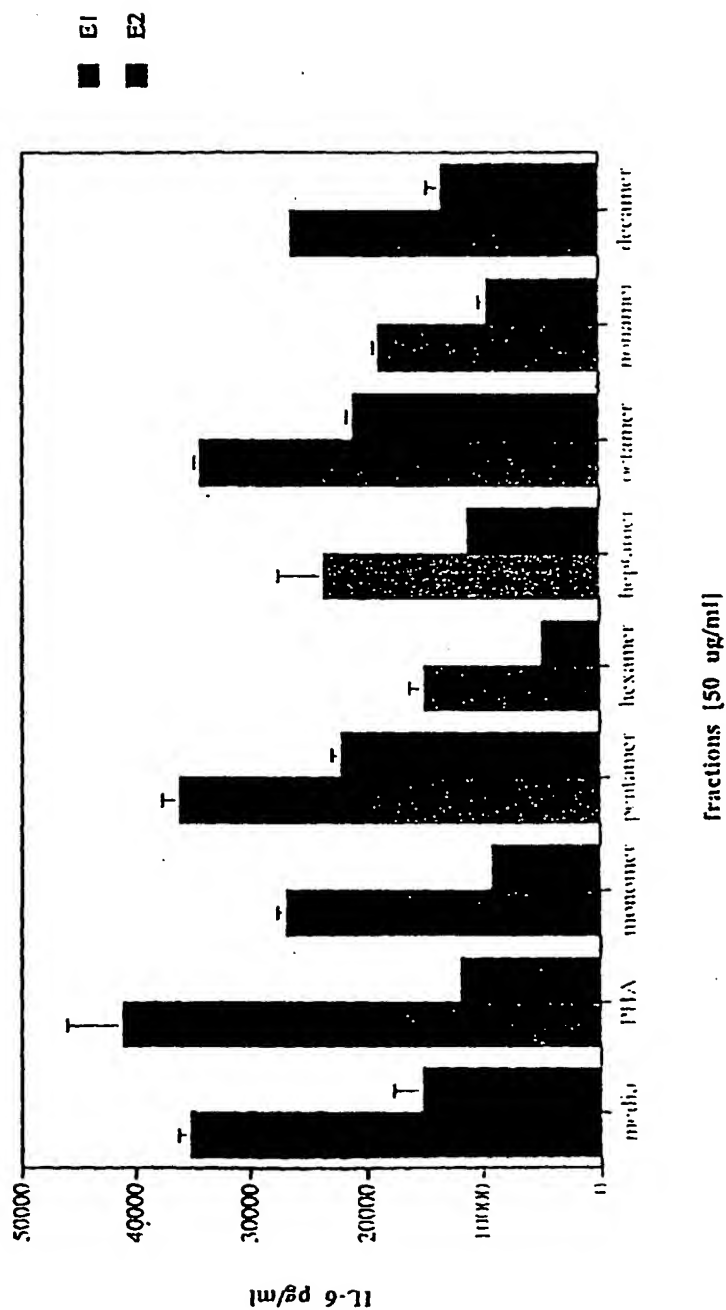
Cocoa [50 ug/ml] + PHA [20 ug/ml]

When the cells were co-stimulated with PHA, there was a general down-regulation of IL-6 expression with the hexamer fraction and above.

The larger oligomers were cytotoxic to the activated cells of some individuals.

FIG. 15B

# Effect of Cocoa Procyanidin Oligomers on IL-6 Secretion (following 72 hr incubation)

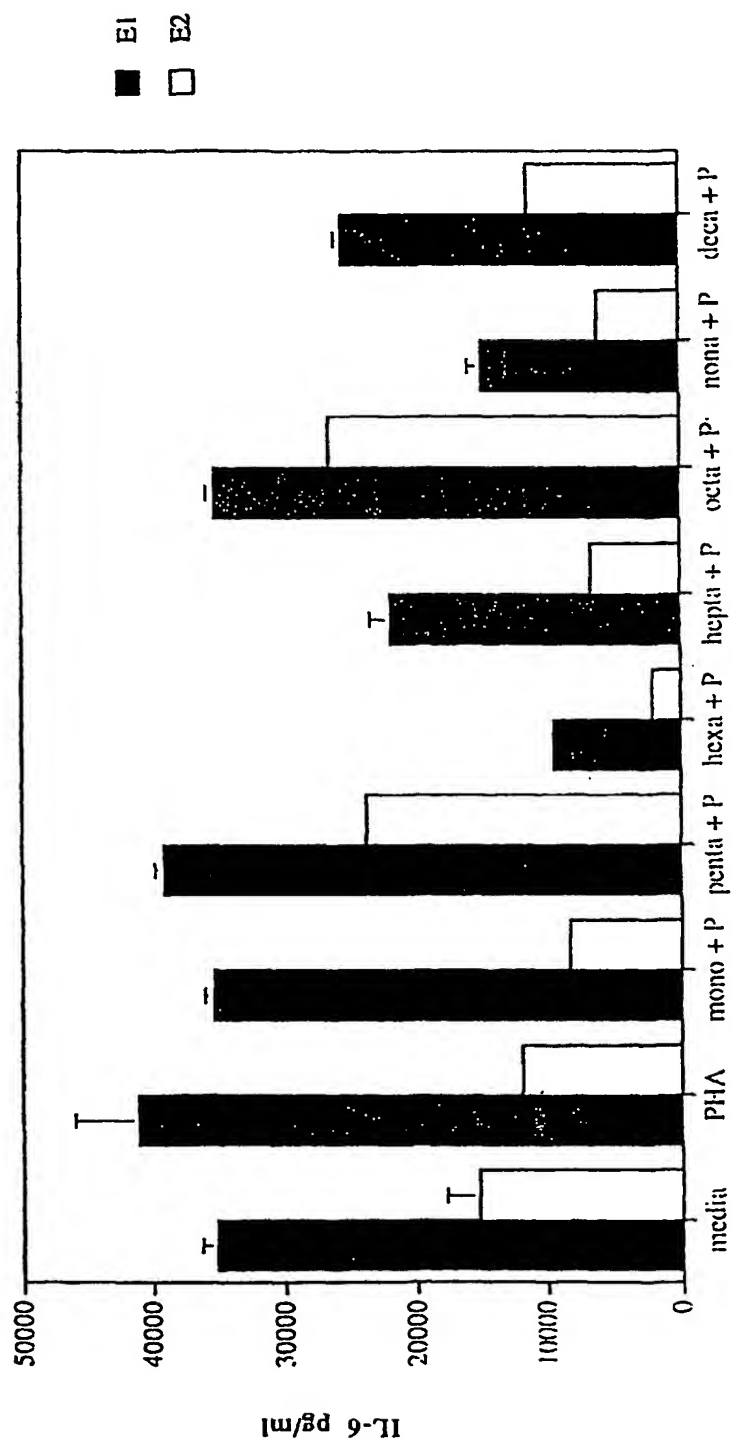


Following stimulation with the cocoa fractions, there was little change in most fractions over the media and PHA controls.

A significant decrease was seen for both subjects with the hexamer and nonamer fractions.

**FIG. 16A**

Effect of Cocoa Procyanidins Oligomers Co-incubated with PHA on IL-6 Secretion (72 Hr)

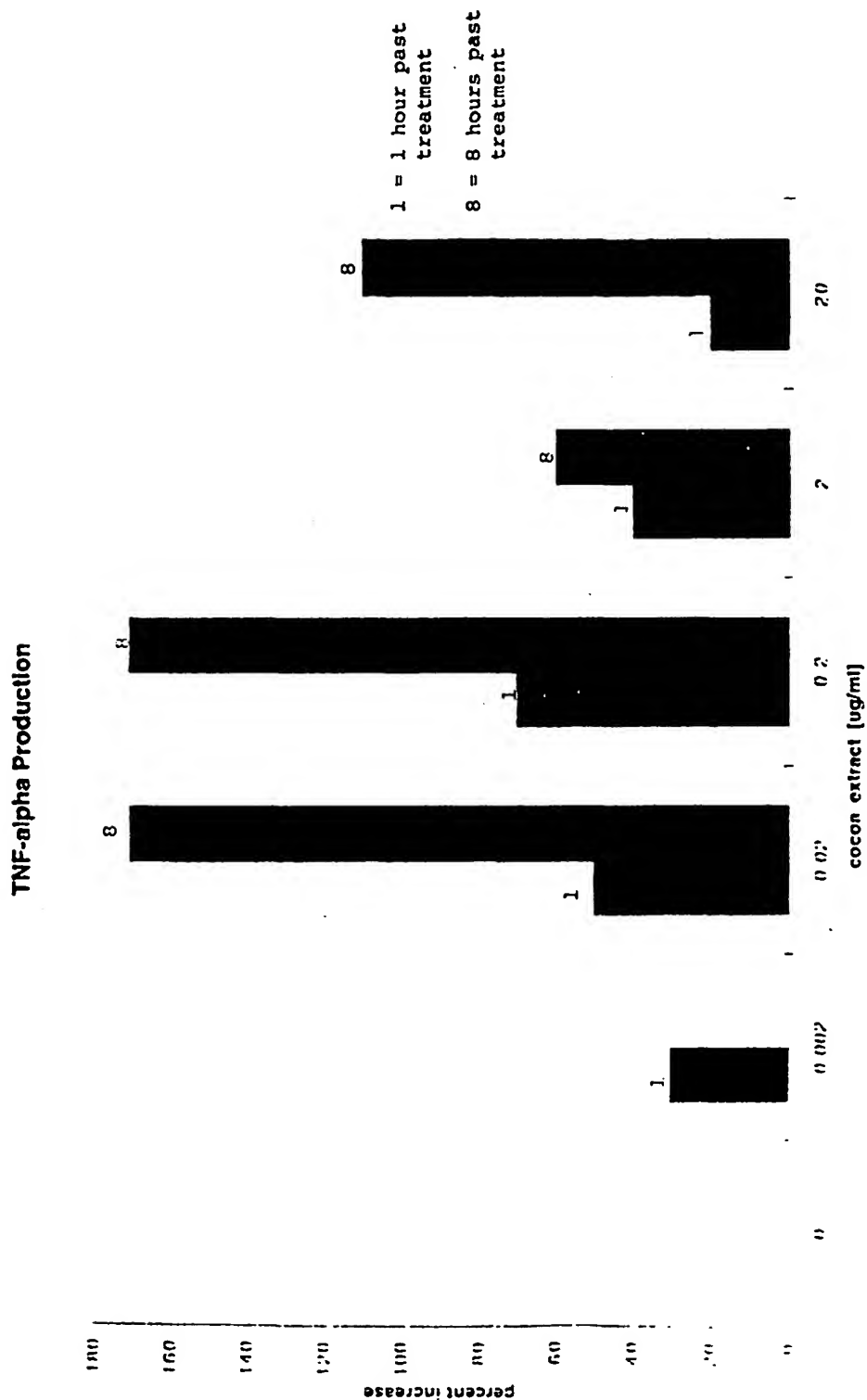


fractions (50ug/ml) + PHA (20 ug/ml)

There was little change in IL-6 with the addition of PHA plus the cocoa extracts.

The hexamer and nonamer both show a significant decrease in IL-6 production.

FIG. 16B



**FIG. 17**

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